For Reference

NOT TO BE TAKEN FROM THIS ROOM

For Reference

NOT TO BE TAKEN FROM THIS ROOM

Ex libris
universitatis
albertaensis

University of Alberta
Printing Department

Thesis
1961(F
#30

THE UNIVERSITY OF ALBERTA

THE EFFECTS OF WATER-SOLUBLE COMPONENTS OF FORAGES ON THE *IN VITRO* CELLULOLYTIC ACTIVITY OF RUMEN MICROORGANISMS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ANIMAL SCIENCE

BY

RONALD RALPH MARQUARDT

EDMONTON, ALBERTA

AUGUST, 1961

Digitized by the Internet Archive
in 2018 with funding from
University of Alberta Libraries

https://archive.org/details/effectsofwaterso00marq

ABSTRACT

Experiments were conducted to study some aspects of the role of aqueous extracts of forages as media for in vitro cellulose digestion by rumen microorganisms.

Approximately 20% to 30% of the dry matter, 50% of the ash, and 30% to 40% of the protein of 5 hay samples were extracted by water. Less than 4% of the dry matter of wheat straw was extracted under the same conditions. A variety of extraction conditions did not greatly alter the amounts of nutrients extracted from forages within the range studied. Most nutrients went into solution within 20 minutes.

When the fermentation media were inadequate in vitro cellulose digestion was highly correlated with concentrations of washed or rewashed inoculum. However, in vitro cellulose digestion was not greatly affected by the concentration of inoculum when the basal medium was used.

The extracts of 6 very different forages supported measurable cellulose digestion. The supplementation of these forage extracts with urea, PO_4^{---}, and SO_4^{--} singly or in combination increased cellulose digestion. In general, the response to urea supplementation was greater for the extracts of non-legumes than for those of legumes. In contrast, legume extracts responded markedly to supplementation with PO_4^{---} and SO_4^{--}, while extracts of non-legumes responded only slightly. Supplementation of either legume extracts

or non-legume extracts with urea, PO_4^{---}, and SO_4^{--} supported cellulose digestion almost equal to that supported by the basal medium.

The cellulolytic activity of another group of 8 forages was studied. In general, similar results were obtained, although the differential responses with the extracts of legumes vs. non-legumes to supplementation was not as consistant as in the earlier trial.

It was concluded that the aqueous extracts of the forages studied contain several important nutrients for the support of rumen cellulolysis and, when supplemented with PO_4^{---}, SO_4^{--}, and urea, they provide a relatively complete medium for the growth and metabolism of the cellulolytic organisms of the bovine rumen.

ACKNOWLEDGEMENTS

The writer wishes to thank Dr. L.W. McElroy, Chairman of the Department of Animal Science, for placing the facilities of the department at his disposal. The guidance and assistance of Dr. J.M. Asplund, Assistant Professor of Animal Science, during the course of the study and in the preparation of this manuscript, is gratefully acknowledged. The writer is indebted to Dr. R.T. Berg, Associate Professor of Animal Husbandry, for assistance with the statistical treatments used. The assistance of the Agricultural Soils and Feed Testing Laboratory, University of Alberta, in providing some of the forages is acknowledged with thanks.

I would also like to thank my wife, Marie, for encouragement during these studies, and for the typing of this thesis.

This work was supported by a grant from the National Research Council.

TABLE OF CONTENTS

Page

INTRODUCTION 1

REVIEW OF LITERATURE 2

I. Rumen Environment 2

II. Nutritive Requirements of Rumen Bacteria 2

Energy 2

Nitrogen 3

Minerals 4

Miscellaneous Factors 7

III. Substrates 8

IV. Products 9

Fatty Acids 9

Other Products of Rumen Fermentation 10

V. Composition of Water-Soluble Extracts 10

VI. The Artificial Rumen 13

EXPERIMENTAL PROCEDURE

I. Preparation and Analysis of Forage and Forage Extract 15

Preparation of Forages 15

Analysis of Forages 15

Preparation of Aqueous Extracts 15

Analysis of Aqueous Extracts 16

II. *In Vitro* Fermentation Techniques 16

General Procedure 16

Preparation of Inoculum 17

Media 18

Fermentation Procedure 21

Cellulose Determination 22

Page

EXPERIMENT 1

Object 24
Experimental 24
Results and Discussion 24
Summary 27

EXPERIMENT 2

Object 28
Experimental 28
Results and Discussion 28
Summary 34

EXPERIMENT 3

Trial 1

Object 36
Experimental 36
Results and Discussion 36
Summary 38

Trial 2

Object 38
Experimental 38
Results and Discussion 39
Summary 40

Trial 3

Object 40
Experimental 40
Results and Discussion 41
Summary 41

Page

EXPERIMENT 3

Trial 4

Object 43
Experimental 43
Results and Discussion 43
Summary 47

EXPERIMENT 4

Trial 1

Object 49
Experimental 49
Results and Discussion 49
Summary 52

Trial 2

Object 52
Experimental 52
Results and Discussion 54
Summary 60

EXPERIMENT 5

Object 62
Description of Forages 62
Experimental 62
Results and Discussion 62
Summary 69
CONCLUSIONS 71
BIBLIOGRAPHY 73

LIST OF TABLES

REVIEW OF LITERATURE

Table | Page

I Summary of the Optimum and Toxic Concentrations of Various Minerals for Maximum *In Vitro* Cellulose Digestion by Rumen Microorganisms, Hubbert *et al.* (1958a) 6

II Amount and Composition of the Water-Soluble Material from a Sample of Cocksfoot Grass, Waite and Gorrod (1959b) .. 11

III Water-Soluble Carbohydrates of Grasses Cut at Four Stages of Maturity, Waite and Gorrod (1959a) 12

EXPERIMENTAL PROCEDURE

IV Composition of the *In Vitro* Basal Medium, Donefer *et al.* (1960) 19

V Concentration of Nutrients Used to Supplement Forage Aqueous Extracts 20

EXPERIMENT 1

VI Effect of Certain Variables on the Solubility of Dry Matter, Ash, and Protein of Alfalfa Brome 25

EXPERIMENT 2

VII Partial Analyses of Forages 29

VIII Analyses of Aqueous Extracts of Forages 30

IX Per Cent of Certain Forage Nutrients Extracted by Water 33

EXPERIMENT 3

X Effect of Altering Nutrient Concentration and Amount of Inoculum on *In Vitro* Cellulose Digestion 37

XI *In Vitro* Cellulose Digestion as Affected by Certain Nutrients 39

Page

EXPERIMENT 4

XII Cellulolytic Activity of Forage Aqueous Extracts and Basal Medium for 3 Replicates . 50

XIII Analysis of Variance of the Data Reported in Table XII 50

XIV Duncan's Multiple - Range Test of Significance 51

XV Effect of Forage Extract and Supplement for 4 Replicates on *In Vitro* Cellulose Digestion 53

XVI Analysis of Variance of Data from Table XV . 54

XVII Duncan's Multiple - Range Test of Significance 55

XVIII Calculated Comparisons of Cellulolytic Activity of Forage Extracts 57

EXPERIMENT 5

XIX Chemical Composition of 8 Forages 63

XX Per Cent Cellulose Digested as Affected by Forage Extract, Supplement, and Replicate .. 64

XXI Analysis of Variance of Data from Table XX . 65

XXII Duncan's Multiple - Range Test of Significance 66

LIST OF FIGURES

Page

EXPERIMENT 3

Figure

1 Regression Curve for Ml. of Washed Inoculum and Cellulose Digested 42

2 Cellulose Digestion as Affected by Volume of Rewashed Inoculum 45

3 Cellulose Digestion as Affected by Volume of Fresh or Autoclaved Rewashed Inoculum ... 46

EXPERIMENT 4

4 Interaction Between Legumes vs. Non-Legumes and Mineral Supplement on In Vitro Cellulose Digestion 59

EXPERIMENT 5

5 Interaction Between Forage Extracts and Supplement on In Vitro Cellulose Digestion.. 67

INTRODUCTION

Forages represent a very important source of biological energy. However, over 50% of the energy potentially available in forage is in the form of complex carbohydrates such as cellulose and hemicellulose which can not be hydrolyzed by the enzymes produced in mammalian tissue. These compounds are made available through fermentation by microorganisms living symbiotically in the fore-stomach or cecum of various species. Ruminants are the most important group of domestic animals through which the energy of forage is made available for human use. They possess a specialized fore-stomach in which exist ideal conditions for the symbiotic digestion of fibrous feeds, the products of which are readily utilized by the host.

The nutrient requirements of the rumen microflora must be adequate if forages are to be rapidly and efficiently degraded into useful products. These nutrients must be provided by the ration or, to a small extent, by the saliva.

It was assumed that the nutrients in the aqueous extracts of forages are those which are most readily available to the rumen microflora and that the amount and type of nutrients that are soluble would be important factors influencing the extent and rapidity that forages are utilized.

This thesis considers some aspects of the importance of the water soluble portion of forages in ruminant nutrition.

REVIEW OF LITERATURE

I. Rumen Environment

The rumen microorganisms which include many different species, Hastings(1944), and Bryant and Burkey (1953) are maintained under relatively constant environmental conditions. The ingested forage is constantly agitated; optimum pH is maintained by a heavy inflow of highly buffered saliva; anaerobic conditions are maintained under an atmosphere composed chiefly of methane and carbon dioxide, Kleiber *et al*. (1943); and temperature is controlled by the heat of the animal and heat produced by fermentation.

II. Nutritive Requirements of Rumen Bacteria

Energy: Rumen microorganisms have a requirement for energy some of which must be in a form that is readily available. Although if the level of readily available carbohydrates is too great, cellulolytic activity of rumen microorganisms is impaired.

Mills *et al*. (1942) showed that the addition of starch to a ration of urea and hay resulted in increased protein synthesis in the rumen of a heifer. Studies *in vitro* by Pearson and Smith (1943) also pointed to the need for an ample supply of available carbohydrates, such as starch and sugars, for the conversion of urea nitrogen to protein nitrogen. In an extensive study with various carbohydrate materials, Arias *et al*. (1951) demonstrated the need for some readily available carbohydrate to

promote efficient cellulose digestion and urea utilization. Belasco (1956) reported that the extent of urea utilization was slightly greater with starch than with cellulose. Xylan and pectin promoted urea utilization but not to the same extent as did starch. He also found that high levels of starch increased rate of cellulose digestion while high levels of dextrose inhibited cellulose digestion. Hershberger*et al*. (1956) also studied the effect of soluble sugars on *in vitro* cellulose digestion. They found that low levels of soluble carbohydrates stimulated cellulose digestion while high levels of soluble carbohydrates inhibited cellulose digestion.

The role of fat as a source of energy for the digestion of cellulose is not well understood. It has been reported that corn oil and lard lowered cellulose digestion by 40% to 90% *in vitro*, and depressed protein digestion and fatty acid production *in vivo* and *in vitro*, Brooks *et al*. (1954).

Nitrogen: B acterial metabolism is usually associated with a multiplying bacterial population. A source of readily available nitrogen is therefore required for bacterial cellular synthesis.

Belasco (1954) using the artificial rumen technique reported that urea was superior to protein supplements as a source of available nitrogen. The utilization of urea in 1:1 mixtures with various feed proteins was very efficient, and its presence in such mixtures improved cellulose

digestion above that observed with feed proteins alone. He concluded that the availability of nitrogen from protein or urea is of great importance to efficient cellulose digestion.

He reported further that increasing the level of urea, when used as a sole source of nitrogen, resulted in an increase in cellulose digestion as well as in the amount of urea utilized. A level of urea was reached above which the concentration of free ammonia apparently inhibited both urea utilization and cellulose digestion.

Minerals: The important role of inorganic nutrients in stimulating metabolic activity of rumen microorganisms and similarly animal performance is well recognized. The digestibility by steers of a high corn cob ration was increased by the presence of inorganic nutrients found in alfalfa, Burroughs *et al*. (1950). Swift *et al*. (1951) reported that more feed energy was made available to lambs on a high corn cob ration by the addition of alfalfa ash.

Hubbert *et al*. (1958b) used a washed suspension of rumen microorganisms in studying the requirements of rumen microflora for sodium and potassium. The presence of potassium in the fermentation medium was found to be essential for *in vitro* cellulose digestion whereas sodium was without effect in the absence of potassium. A study of the inter relationships of varying concentrations of sodium and potassium showed that the addition of sodium to a

fermentation medium containing 50 mcg. or less of potassium per ml. had either no influence or depressed cellulose digestion. However, when the potassium concentration was from 100 mcg. to 400 mcg. per ml. additions of sodium increased cellulose digestion. They concluded that 100 mcg. of potassium per ml. of fermentation medium is essential for _in vitro_ cellulose digestion. Although sodium is not essential in the strictest sense, it appears desirable to maintain a certain ratio between the concentration of sodium and potassium for maximum cellulose digestion.

Rumen microorganisms require phosphorus for efficient cellulose digestion, Burroughs _et al_. (1951). _In vitro_ cellulose digestion increased from 34% in a basal medium, to 80% when 80 mcg. per ml. of standard phosphorus medium was added to the artificial rumen, Anderson _et al_. (1956).

Hubbert _et al_. (1958a), studied rumen microorganism requirements for sulphur, magnesium, calcium, manganese, iron, copper, cobalt, zinc, and boron. The range of concentration of the minerals found to support maximum _in vitro_ cellulose digestion is summarized in Table I.

Of the minerals studied, sulphur, magnesium, and calcium were found to be the inorganic nutrients most likely to be deficient in a prepared fermentation medium. Additions of manganese, iron, copper, cobalt, zinc, and boron to the fermentation medium did not increase cellulose digestion.

The concentration of sulphur, magnesium, calcium, manganese, and iron found to depress cellulose digestion was very high. Extremely low levels of copper, cobalt, zinc, and boron depressed cellulose digestion.

Hubbert *et al*. (1958a) were unable to demonstrate a cobalt requirement for rumen microorganisms *in vitro*, however, the effects of cobalt deficiency *in vivo* are quite well established. Gall *et al*. (1949) showed that there was a reduced and altered rumen bacterial population in cobalt deficient sheep. Whether this was due to the altered physiology of the animal or to a nutrient deficiency of the rumen microflora has not been established.

TABLE I

SUMMARY OF THE OPTIMUM AND TOXIC CONCENTRATION OF VARIOUS MINERALS FOR MAXIMUM *IN VITRO* CELLULOSE DIGESTION BY RUMEN MICROORGANISMS, HUBBERT *ET AL*. (1958a)

Element	Form Added	Optimum Elemental Concentration mcg./ml.	Toxic Elemental Concentration mcg./ml.
S	Na_2SO_4	10 - 500	1000
Mg	$MgSO_4$	20 - 160	320
Ca	$CaCl_2$	50 - 300	450
Mn	$MnSO_4 \cdot H_2O$	0 - 160	320
Fe	$FeSO_4 \cdot H_2O$	0 - 50	300
Cu	$CuSO_4 \cdot 5H_2O$	0 - 1	1.5
Co	$CoCl_2 \cdot 6H_2O$	0 - 0.5	5.
Zn	$ZnSO_4 \cdot 7H_2O$	0 - 0.05	5.
B	$Na_2B_4O_7 \cdot 10H_2O$	0 - 0	0.5

The inability to remove trace elements from bacterial inoculum completely would tend to mask in vitro rumen microorganism requirements for these elements. Perhaps some of the elements, which failed to stimulate bacterial activity in the studies of Hubbert et al. (1958a and 1958b) were present in the bacterial suspension in sufficient amounts to meet rumen microorganism requirements, as a result requirements for those elements would not be established.

Bryant et al. (1959) studied the nutrient requirements of Bacteroides succinogenes which is one of the more prominent cellulolytic species in the rumen. When phosphorus, potassium, magnesium, ammonia, or copper was omitted from the otherwise complete medium, little or no bacterial growth occurred. Iron, cobalt, and manganese had no effect on growth indicating that they were not essential or that the requirements for these elements were met by the introduction of trace amounts of elements into the medium. The sulphur requirement was not adequately studied.

Miscellaneous Factors: Certain fatty acids appear to be specifically required by the rumen microflora. Bentley et al. (1955) reported that 5- and 6- carbon volatile fatty acid and iso-butyric stimulated the growth of rumen microorganisms in vitro as determined by increased cellulose digestion and non-protein nitrogen assimilation. Bryant and Doetsch (1955) found that a combination of

straight chain and branched chain 5- and 6- carbon fatty acids was required for the growth of the cellulolytic rumen organism, Bacteroides succinogenes. Cline et al. (1958) reported that valeric acid could be synthesized from starch and it appeared that valeric acid was required for bacterial synthesis of protein.

It has also been found biotin and p-aminobenzoic acid are active in promoting cellulose digestion by washed suspensions of mixed rumen organisms, Bentley et al. (1954).

III. Substrates

Ruminants are basically forage consumers and digest large amounts of cellulose and other complex carbohydrates. Since mammalian tissues have no cellulolytic capacity the process of cellulose digestion in the ruminant is entirely dependent upon the cellulases produced by rumen microorganisms.

Enzymes present in the rumen hydrolyze various complex carbohydrates to their monosaccharide units. Cellulose appears to go through tetrose and cellobiose intermediates to glucose, Hash et al. (1954). Starch is converted to glucose, Kitts et al. (1954). Fructosans are hydrolyzed by the enzymes found in grass and those secreted by the holotrich protozoa but the invertase activity of bacteria is negligible, Thomas (1960). Rumen protozoa possess enzymes which hydrolyze pectic substances to galacturonic acid and low oligouronides. The role played by protozoa in pectic digestion has not been established, but it is unlikely that they are the sole agents responsible

for degradation in the rumen, Wright (1959). Fermentation of pentosans by rumen microorganisms _in vitro_ has been demonstrated, Howard (1955).

The products of hydrolysis of complex carbohydrates (glucose, fructose, galacturonic acids, pentoses, etc.) are rapidly converted by rumen microorganisms to short chain fatty acids.

Waite and Gorrod (1959b) reported the digestibility of the soluble sugar fraction of grasses to be 99%.

IV. Products

Fatty Acids: The total volatile fatty acids in rumen contents range from two to five grams per 100 grams of dry matter when expressed as acetic acid, Elsden (1946) and Gray (1951). It has been estimated that 12.1 therms of volatile fatty acids were produced per day in the rumen of 1500 lb. Holstein steers consuming a ration of hay, Stewart _et al_. (1958). This accounted for 41.7% of the digested calories and 23.3% of the gross energy of the ration.

The quantitatively most important acids occurring in the rumen are acetic, propionic, and butyric, which occur in the proportions of about 65:20:10, respectively. These ratios are not fixed and can be shifted by altering the composition of the physical form of the ration, i.e., pelleting, fine grinding, and steam treatment of starch. It has been postulated that the evaluation of a ration for a specific purpose, i.e., the production of flesh, milk, or

other products, can be made on the basis of the proportion of the various volatile fatty acids resulting from its fermentation in the rumen, Shaw (1959).

Longer chain fatty acids, as well as shorter chain formic acid, are present in the rumen only in small amounts. Valeric acid represents from 1% to 4% of the total rumen fatty acids, Annison (1954). Six carbon fatty acids are less commonly found.

Although volatile fatty acids are formed mainly from the degradation of cellulose, starch, and carbohydrates, smaller amounts are formed from the deamination of amino acids, Annison and Lewis (1959).

Other Products of Rumen Fermentation: Proteolytic enzymes in the rumen rapidly degrade most of the ingested protein to peptides, amino acids, and ammonia. These non-protein nitrogenous substances are utilized for the synthesis of microbial protein, McDonald (1952). This microbial protein, with biological values of around 80, can be digested and utilized by the ruminant, Annison and Lewis (1959).

It has been demonstrated that most of the known B-complex vitamins, as well as vitamin K are synthesized in the rumen. The ruminant is therefore not dependent upon exogenous sources of these vitamins, Kon *et al*. (1947).

V. Composition of Water Soluble Extracts:

Waite and Gorrod (1959b) reported that 26.3% of a cocksfoot grass was soluble in water at 60°C. The amount and composition of the material extracted from

cocksfoot grass is given in Table II.

TABLE II

AMOUNT AND COMPOSITION OF THE WATER-SOLUBLE MATERIAL FROM A SAMPLE OF COCKSFOOT GRASS, WAITE AND GORROD (1959b)

(g./100g. Moisture-Free Original Grass)

Total water-soluble extract			26.3
Composition:			
Ash			9.4
Carbohydrates:	Hexoses	1.4	
	Sucrose	3.2	
	Fructosan	1.2	
	Galactan	0.8	6.6
Organic acids			5.2
N X 6.25			2.5
Phenols			1.0
Unidentified			1.0
Unaccounted for			0.6

A study of the effect of temperature on the amount of water-soluble material extracted showed an increase from 16.3% after 30 minutes at 4°C to 18.0% at 60°C to 20.0% at 100°C in one sample. The increase at 60°C came very largely from the ionic fraction of the forage. At 100°C, however, there was also an appreciable increase (15%) in the non-ionic fraction, Waite and Gorrod (1959b).

Twenty-five to 30% of total forage nitrogen consists of non-protein nitrogen which is mainly amides, ammonia, nitrate, and choline, Annison and Lewis (1959). It is likely that this highly soluble non-protein fraction of

forages is a major component of the nitrogen fraction of forage aqueous extracts.

The soluble carbohydrates content of grasses showed a seasonal variation, with a peak occurring prior to flowering. After flowering there was a gradual decline in the level of soluble carbohydrates. The variation between forages and between seasons in the solubility of forage carbohydrates is illustrated in Table III.

TABLE III

WATER-SOLUBLE CARBOHYDRATES OF GRASSES CUT AT FOUR STAGES OF MATURITY, WAITE AND GORROD (1959a)

		As % of Dry Matter			
		June 21	July 1	July 18	Aug. 12
Rye-grass	Western Wolths	14.5	12.5	18.2	4.4
Rye-grass	S 24	17.7	31.5	20.6	1.8
Rye-grass	S 23	15.8	27.1	17.7	4.2
Timothy	S 48	19.9	19.7	14.2	11.4
Cocksfoot	S 143	12.0	14.1	11.6	0.5

In addition to a seasonal and species variation in total amount of water-soluble carbohydrates there is also considerable seasonal and species variation in the proportions of the main types of soluble carbohydrates. The major portion of the water-soluble carbohydrates of grasses is composed of fructosan. Sucrose, which is generally much lower in concentration, is the next most abundant carbohydrate. The remaining portion is composed of small

amounts of galactan and other hexoses, Waite (1957).

Unlike grasses, alfalfa does not possess large amounts of reserve polysaccharides in the form of fructosan, but does contain a small amount of a water-soluble glucosan and a considerable amount of pectin, Hirst _et_ _al_. (1959).

Cason _et_ _al_. (1955) reported that the cellulolytic activity* in fresh forage aqueous extracts compared to that of bovine rumen contents. He concluded that the activity associated with forages could contribute appreciably to total cellulolytic activity in the rumen.

VI. The Artificial Rumen

The _in_ _vitro_ incubation of rumen contents with specific substrates in various types of fermentation apparatus has been studied for several years.

The basic design of these artificial rumens has been of three types:

1. Impermeable systems, in which rumen contents were incubated with specific substrates, Pearson and Smith (1943), Marston (1948), Gray _et_ _al_. (1951), Ruf _et_ _al_. (1953), Bentley _et_ _al_. (1954), Cheng _et_ _al_. (1955), Barnett (1957), and Donefer _et_ _al_. (1960).

---

* The term "cellulolytic activity" in this thesis will refer to completeness of certain nutrient medium in supplying the nutrients required for the _in_ _vitro_ digestion of cellulose.

2. Permeable systems in which rumen contents were incubated with specific substance, the fermentation being dialyzed against mineral solutions of varying compositions, Louw _et_ _al_. (1949), Wasserman _et_ _al_. (1952), Huhtanen _et_ _al_. (1954), and Asplund _et_ _al_. (1958).
3. Continuous control systems in which a high degree of control can be exercised over the progress of _in_ _vitro_ fermentation, Davey _et_ _al_. (1960).

El-Shazly _et_ _al_. (1960) compared these three types of artificial rumens. Purified wood cellulose and several types of hay were used as substrates, with cellulose digestion, total volatile fatty acid production and ammonia nitrogen production being used as criteria of microbial activity. He concluded that since no major differences were found between different types of apparatus, that the all glass apparatus appeared to be advantageous because of its simplicity.

EXPERIMENTAL PROCEDURE

1. Preparation and Analysis of Forage and Forage Extract

Preparation of Forage

Some of the forages were obtained from the University of Alberta Livestock Farm. These forages were ground in a Wiley mill through a 20 mesh screen and stored under refrigeration at 35°F. The other forages were obtained from the Agricultural Soils and Feed Testing Laboratory at the University of Alberta.

Analysis of Forages

The forages reported in Experiment 2 were analyzed for moisture, protein (N X 6.25), cellulose, ash, calcium, and phosphorus.

Moisture, protein, and ash analyses were performed using the methods recommended by the Association of Official Agricultural Chemists (1955). Calcium was determined by the E.D.T.A. -- cal red method, and phosphorus by the ammonia-metavandate method as outlined by Jackson (1958).

The analyses for moisture, protein, crude fiber, calcium, and phosphorus of the forages used for Experiment 5 were obtained from the Agricultural Soils and Feed Testing Laboratory at the University of Alberta.

Preparation of Aqueous Extracts

Routinely hay and distilled water were mixed in the weight to volume ratio of 20:1, and the forage-water mixture was allowed to stand 3 hours, with occasional

stirring, at room temperature (approximately 68°F). The aqueous extract was separated from the residue mixture through a 20.0 cm. #4 Whatman filter paper supported by a Buchner funnel. The filtrate was stored until required, in plastic containers in a refrigerated room maintained at -3°F.

Analyses of Aqueous Extracts

The 8 forage extracts used in Experiment 5 were not chemically analyzed.

Forage extracts used in the other experiments were analyzed for dry matter, protein (N X 6.25), and ash. Calcium, phosphorus, organic acids, and water-soluble carbohydrates values were also determined for the extracts of 6 forages, Table VIII. Chemical analyses of the forage extracts for dry matter, ash, calcium, and phosphorus were similar to those used for the forages, with the exception that sample aliquots were first evaporated to dryness on the steam bath before standard procedure was started. Twenty ml. aliquots were used for protein analysis. Water-soluble carbohydrates were estimated by the anthrone method, and organic acids were estimated by the continuous ether extraction method, Neish (1952).

II. *In Vitro* Fermentation Techniques

General Procedure

Cellulose digestion *in vitro* was determined by the *in vitro* technique developed at the Ohio Agricultural Experimental Station and described by Quicke *et al*. (1959),

with slight modification, Donifer _et_ _al_. (1960). This modified technique is described as follows:

The _in_ _vitro_ system consisted of fermentation tubes (90 ml., pyrex 8260) each equipped with rubber stopper and gas inlet tube through which CO_2 was passed throughout the fermentation period at a rate of approximately 160 bubbles per minute. Gas was exhausted by way of the clearance between the pouring lip of the tube and the rubber stopper. Total liquid volume in each tube was 55 ml., with 200 mg. of purified cellulose (Solka Floc SW 40 A) as substrate.

Preparation of Inoculum

A rumen fistulated Holstein cow, fed good quality alfalfa hay, served as the source of rumen ingesta.

The ingesta was pressed through several layers of cheese cloth, and 4 lbs. of the resultant solid pulp was extracted with 1000 ml. of phosphate buffer (pH 7), according to the method of Johnson _et_ _al_. (1958). Preliminary to making the extract, the phosphate buffer solution (1.059 gm. Na_2HPO_4 + 0.436 gm. KH_2PO_4 per liter) was preheated to 45°C to compensate for drop of temperature to approximately 40°C during extracting procedure, saturated with CO_2 by rapidly bubbling CO_2 through the solution for 35 minutes, and re-adjusted to pH 7 with Na_2CO_3 to compensate for the pH drop due to CO_2 saturation. After moderate agitation the pulp and phosphate buffer were re-pressed, and the resultant phosphate buffer extract (PBE)

transported to the laboratory in a thermos container. The sediment from 1000 ml. of PBE obtained by centrifugation at approximately 30,000 r.p.m. was usually resuspended in 800 ml. of distilled water and recentrifuged. The washed or rewashed sediment was then resuspended, with the aid of a M S E Nelco 10 blender, in 250 ml. of water. This was the bacterial inoculum. The distilled water used for resuspending the bacteria contained 25 mg. cysteine HCl, was saturated with CO_2 and maintained at 40°C. Only the sediment above a line one inch from the bottom liner was used to make up the final bacterial suspension.

Media

Control samples were included in each run in which a complete basal medium was used, Donefer et al. (1960). The composition of this medium is given in Table IV and hereinafter will be referred to as the basal medium.

The average in vitro digestion of cellulose supported by the basal medium during the seven, 24- hour and the six, 44- hour fermentation periods was 93.7 ± 1.3%* and 98.6 ± 0.8%* respectively. These values are similar to those reported by Donefer et al. (1960).

Varying concentrations of the basal medium were also used.

* Standard deviation, between runs.

TABLE IV

COMPOSITION OF THE IN VITRO BASAL MEDIUM, DONEFER ET AL. (1960)

Solution	Volume used per tube	Amount used per tube
	ml.	mg.
Mineral mixture[a]	10.0	(see a)
Iron and Calcium ($FeCO_3.6H_2O$ 4.4 mg./ml.	0.5	2.200
$CaCl_2.2H_2O$ 5.29 mg./ml.)	---	2.645
Glucose (100 mg./ml.)*	0.5	50
Urea (126 mg./ml.)*	0.5	63
Biotin (10 mcg./ml.)	1.0	10 mcg.
p-Aminobenzoic acid (100 mcg./ml.)	0.25	25 mcg.
n-Valeric acid (5 mg./ml.)*	3.0	15
Casein hydrolysate-enzymatic* (20 mg./ml.)	2.5	50
Na_2CO_3 (200 mg./ml.)	1.5	300
Phosphate buffer (pH 7)[b]	10.0	(see b)

a Na_2HPO_4, 5.65 gm.; $NaH_2PO_4.H_2O$, 6.27 gm.; KCl, 2.15 gm.; NaCl, 2.15 gm.; $MgSO_4.7H_2O$, 0.582 gm.; and Na_2SO_4, 0.75 gm.: per liter.

b $NaHPO_4$, 1.059 gm.; KH_2PO_4, 0.436 gm.; per liter.

* Prepared prior to each fermentation run.

The nutrient media for the majority of the experiments consisted of forage aqueous extracts which were supplemented, in some experiments, with urea, phosphate (PO_4^{---}), and sulphate (SO_4^{--}), either singly or in combination. The concentration of nutrients added per digestion flask is given in Table V.

TABLE V

CONCENTRATION OF NUTRIENTS USED TO SUPPLEMENT FORAGE AQUEOUS EXTRACTS

	Amount used per tube	Volume per tube
	gm.	ml.
Urea	0.063	5
PO_4^{---} as Na_2HPO_4	0.038	10
and NaH_2PO_4	0.042	
SO_4^{--} as Na_2SO_4	0.010	
Na_2CO_3	0.200	1

Since the nutrients supplied by a complete control medium were more than double those required for complete digestion of 0.2000 gm. of purified cellulose, Kamstra *et al.* (1958), only two-thirds of the PO_4^{---} and SO_4^{--} levels reported by Donefer *et al.* (1960) were used to supplement forage aqueous extracts. It was assumed that this would tend to reduce any nutrient imbalance that might be created by the addition of these nutrients to an incomplete nutrient medium.

Fermentation Procedure

The nutrient medium was pipetted into fermentation tubes containing Solka Floc. Distilled water was added to bring the final volume of medium plus inoculum to 55 milliliters.

The tubes were immediately placed in a water bath thermostatically controlled at 40°C. Two drops of mineral oil were added to each tube in order to prevent foaming, after which the tubes were connected to a CO_2 source. The inoculum, when prepared, was added to the fermentation tubes and the fermentation period was initiated. Fermentation periods of either 24 or 44 hours were used. When the bacterial suspension was washed, a 24 hour fermentation period was used, whereas, when the bacterial suspension was rewashed, a 44 hour period was used.

The 24 hour *in vitro* cellulose digestion supported by the basal medium was over 90% with washed inoculum, and only 40% with the rewashed inoculum. It was concluded that rewashing the bacterial suspension increased the lag time for initiation of rapid cellulose digestion. The fermentation period was therefore extended when the bacterial suspension was rewashed.

At the end of the *in vitro* fermentation the tubes were disconnected and material adhering to the CO_2 inlet tube was washed back into the centrifuge tube with distilled water. Material adhering to the sides of the centrifuge tubes was loosened by rubbing with a rubber

policeman and the insoluble material was sedimented at 2,200 r.p.m. for 8 minutes in M S E "Major Refrigerator", centrifuge. The supernatant was decanted, care being taken not to disturb the solid material at the bottom of the tube.

Cellulose Determination

Residual cellulose was determined by a modification of the Crampton and Maynard (1938) method which is described as follows:

Twenty ml. of 80% acetic acid and 2 ml. of concentrated nitric acid were added to each tube. Glass stirring rods were inserted into each tube and the contents were completely mixed. The tubes were then placed in a wire basket and were left in a boiling water bath for 30 minutes. At the end of the digestion period the tubes were removed from the water bath and allowed to cool for 5 minutes. Twenty-five ml. of 95% ethanol was added to each tube after which the contents were filtered through Selas crucibles (extremely coarse porosity). The tube contents were quantitatively transferred to a crucible using a wash bottle filled with 95% ethanol. The precipitate in the crucible was washed with approximately 5 ml. of acetone. The crucible was dried and weighed followed by ashing in the muffle furnace and reweighing. The loss of weight on ashing was equal to the amount of cellulose left. The difference between the initial cellulose content of the forage and the residual cellulose

after fermentation was taken as a measure of the cellulose digested.

Bacterial protein content of the inoculum was estimated from trichloroacetic acid insoluble nitrogen. The method of analysis has been previously described by Cline *et al.* (1958).

Statisical methods were those described by Snedecor (1956) and Duncan (1955).

EXPERIMENT 1.

Object: To determine the influence of volume and temperature of water, duration of extraction, grinding, and storage period on the solubility of forage dry matter, protein, and ash.

Experimental: Alfalfa brome hay was used to characterize the solubility of the dry matter, ash, and protein of forage. Except for the factor considered all other variables were held constant according to method described in Table VI. Variables considered were temperature, 20°C, 37°C, and 90°C; length of extraction, 20 minutes, 150 minutes, and 24 hours; water to forage ratio, 10:1, 20:1, and 40:1; date of extraction, May, 1960, and April, 1961; and the preparation of forage, coarse grinding, fine grinding, and ball milling.

Results and Discussion: The results for this experiment are given in Table VI.

The maximum relative variation between treatments in amount of dry matter, ash, and protein extracted from the forage was only 9.6, 10.3, and 15.3% respectively.

The amount of dry matter, protein, and ash extracted at 20 minutes was over 94% of the amount extracted at 150 minutes.

Leaving the solution-forage mixture over a 24 hour period resulted in a reduction of dry matter solubility and a small increase in ash and protein solubility. The decreased dry matter recovery was likely caused by

TABLE VI

EFFECT OF CERTAIN VARIABLES ON THE SOLUBILITY OF DRY MATTER, ASH, AND PROTEIN OF ALFALFA BROME

Variable	Time Hrs.	Temp. °C	Ratio of H_2O to Forage (Vol:Wt)	Relative Dry Matter Solubility*		Relative Ash Solubility*		Relative Protein Solubility*	
				Mean	S.D.	Mean	S.D.	Mean	S.D.
Duration of Extraction:									
	0.3	20	20:1	97.9	0.8	98.0	3.4	94.0	2.0
	2.5	20	20:1	100.0	0.5	100.0	3.3	100.0	1.4
	24	20	20:1	95.2	1.1	108.0	3.7	105.0	0.8
Temperature of Extraction:									
	2.5	20	20:1	100.0	0.5	100.0	3.3	100.0	1.4
	2.5	37	20:1	100.3	0.7	104.0	2.1	101.0	0.8
	2.5	90	20:1	104.8	0.3	109.0	3.0	94.2	0.2
Water Concentration:									
	2.5	20	10:1	98.4	0.9	100.0	5.1	97.5	0.6
	2.5	20	20:1	100.0	0.5	100.0	3.3	100.0	1.4
	2.5	20	40:1	100.3	0.7	99.1	5.4	101.0	1.4
Date:									
Apr./61	2.5	20	20:1	100.0	0.5	100.0	3.3	100.0	1.4
May /60	2.5	20	20:1	99.0	0.3	98.7	2.0	102.0	0.8
Preparation of Forage:									
Coarse	2.5	20	20:1	97.6	0.7	96.5	1.6	89.7	0.8
Fine	2.5	20	20:1	100.0	0.5	100.0	3.3	100.0	1.4
Ball Mill	2.5	20	20:1	97.3	0.7	103.0	2.4	91.3	0.7

* For purposes of comparison the extract from fine ground forage, extracted in April, 1961, for 3 hours at 20°C with 20 volumes of H_2O was taken as 100.0.

S.D. Standard deviation.

fermentation of soluble sugars.

Increasing the temperature from 20°C to 37°C to 90°C resulted in successive increases in solubility of dry matter and ash. The relative amounts of ash and dry matter extracted at 20°C as compared to 90°C increased by 9% and 4.8% respectively. Solubility of protein decreased at 90°C as compared to 37°C. It is assumed that at 90°C some protein denaturation occurred thereby reducing protein solubility.

The ratio of water to forage used for the solution of the hay sample and forage storage period had little effect on dry matter, ash, or protein solubility.

Breaking the hay sample into short pieces by passing it through the Wiley mill without a screen as compared to passing the forage through a 20 mesh screen reduced the solubility of dry matter, ash, and protein of forage.

There was less dry matter and protein extracted from forage which had been ball milled for 8 hours than from the finely ground sample. The protein level was reduced by 8.8%. The solubility of the ash fraction, on the other hand, was increased.

The addition of considerable quantities of phosphate buffer at pH of 6.8 was ineffective in altering the 6.2 pH of the forage extract. It appears that the pH of the forage extract is slightly acidic and that forage extracts possess some buffer capacity.

Summary:

1. The major portion of forage water-soluble nutrients went into solution within 20 minutes.
2. Increasing the temperature of extraction resulted in small increases in recovery of ash and protein, and small decreases in recovery of protein from forages.
3. Ratio of water to forage and duration of forage storage had little effect on the solubility of forage constituents.
4. Coarse grinding and ball milling tended somewhat to reduce recovery of dry matter, ash, and protein from forages.
5. Although the solubility of certain forage constituents were slightly influenced by temperature of water, duration of extraction, method of grinding, or date of extraction; it was not possible to alter greatly the amounts of forage constituents extracted within the range studied, by these factors.

EXPERIMENT 2

Object: To determine the partial composition of 6 forages and to quantitatively account for the dry matter from aqueous extracts of these forages.

Experimental: The dry matter, ash, protein, calcium, phosphorus, and cellulose content of 6 forages and the dry matter, ash, protein, phosphorus, carbohydrate, and ether extract content of the aqueous extract of these forages were determined.

Results and Discussion: The partial analyses of 6 forages are given in Table VII. The composition of the forage aqueous extracts, expressed in grams soluble nutrients per 100 grams of forage, is given in Table VIII.

The sum of the weights of the extract constituents determined did not account for 100% of the extracted dry matter, Table VIII. The quantitative estimation of forage extract constituents was high for non-legumes and low for legumes. It was assumed that this discrepancy was partially caused by inaccurate carbohydrate estimates and high protein values.

The intensity of color produced by the reaction of anthrone reagent with a given amount of various carbohydrates is relatively constant, although with some sugars this is not true. Galacturonic acid, a major constituent of legume extract, gave only 4% of the color yield of glucose, Deriaz (1961). The color yield of some other hexoses is given by Morris (1948).

TABLE VII

PARTIAL ANALYSES OF FORAGES

Composition	Alfalfa 1		Alfalfa Brome		Alfalfa 2		Native Hay		Wheat Straw		Tame Hay	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
% Moisture	7.18	0.03	6.54	0.11	9.07	0.07	7.48	0.10	6.51	0.01	7.46	0.16
% Protein	14.78	0.16	11.80	0.16	16.23	0.08	9.79	0.26	2.00	0.01	8.35	0.04
% Ash	6.68	0.04	6.77	0.04	7.35	0.01	5.73	0.06	3.38	0.04	5.19	0.01
% Calcium	1.92	0.01	1.11	0.01	1.89	0.00	0.37	0.01	0.17	0.01	0.70	0.01
% Phosphorus	0.12	0.00	0.13	0.00	0.15	0.00	0.15	0.00	0.02	0.00	0.12	0.00
% Cellulose	28.37	0.42	29.42	0.45	29.22	0.73	30.96	0.50	44.26	0.04	28.30	0.04

S.D. Standard deviation.

TABLE VIII

ANALYSES OF AQUEOUS EXTRACTS OF FORAGES

Composition (gm. of water-soluble nutrients per 100 gm. of forage)	Alfalfa 1		Alfalfa Brome		Alfalfa 2		Native Hay		Wheat Straw		Tame Hay	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
1. Dry matter	26.35	0.03	24.35	0.07	21.72	0.00	18.87	0.06	3.61	0.04	23.36	0.40
2. Ash	4.35	0.22	4.46	0.06	3.78	0.06	3.17	0.20	0.57	0.01	3.03	0.30
3. Protein	5.59	0.07	4.95	0.07	6.22	0.13	2.86	0.12	0.91	0.03	3.32	0.09
4. Calcium	1.06	0.03	0.70	0.01	0.68	0.01	0.20	0.01	0.04	0.01	0.34	0.01
5. Phosphorus	0.003	0.000	0.007	0.001	0.004	0.001	0.005	0.000	0.002	0.000	0.007	0.001
6. Carbohydrates	6.6	0.1	9.2	0.0	3.7	0.0	10.6	0.1	1.0	0.0	15.8	0.2
7. Ether extract (organic acids)	4.9	0.1	3.7	0.06	6.3	0.6	4.2	0.2	2.0	0.1	8.3	0.0
8. Total 2 through 7	22.6		23.1		20.7		21.1		4.5		30.7	
9. Recovery 8 as % of 1	85.6		94.7		95.4		111.6		125.0		131.2	

S.D. Standard deviation.

Since the composition of grass water-soluble carbohydrates is not the same as legume water-soluble carbohydrates, Waite (1957) and Hirst (1959), variable results can easily arise when the anthrone method is used to determine total water-soluble carbohydrates.

The major portion, approximately 50% or more, of forage extract was composed of carbohydrates and organic acids. The remaining portion was largely accounted for by ash and protein. Protein levels were slightly higher than ash for all forage extracts except for tame hay extract.

The amount of certain forage nutrients that are extracted in water is given in Table IX.

The amount of dry matter extracted from the 5 hays ranged from a high of 28.4% for alfalfa 1, to a low of 20.4% for native hay. Only 3.9% of the dry matter of wheat straw was extracted.

The amount of ash extracted from the 5 hays ranged from a high of 65.9% for alfalfa brome, to a low of 51.4% for alfalfa 2. Only 16.9% of the ash content of wheat straw was extracted.

The major portion of the forage phosphorus was not extracted. The amount of phosphorus extracted from the 5 hays ranged from a high of 5.8% to a low of 2.5%. Ten per cent of the phosphorus content of wheat straw was extracted.

The amount of calcium extracted from all 6 forages

ranged from a high of 63.1% for alfalfa brome, to a low of 23.5% for wheat straw.

The amount of protein (N X 6.25) extracted from the 6 forages ranged from a high of 45.5% for wheat straw, to a low of 29.2% for native hay.

It can be seen that there is considerable variation between species and within species in the amounts of forage components that are extracted by water.

Forage extracts of most legumes, like the forages from which they were extracted , tend to be high in protein and calcium. The composition of the water-soluble carbohydrates fraction of grass is different from that of legumes. Grasses also, tend to have larger concentrations of water-soluble carbohydrates than do legumes.

Two important groups of nutrients required for rumen cellulolytic bacteria are nitrogenous compounds and minerals. The high solubility of forage ash and nitrogen indicates that water extracts of forages are able to provide part of the nitrogen and some of the minerals required by rumen microflora. Although a large portion of the ash of forages is soluble, the solubility of all essential minerals may not be equally as high. This was demonstrated with phosphorus, the solubility of which was much lower than that of calcium or of ash.

TABLE IX

PER CENT OF CERTAIN FORAGE NUTRIENTS EXTRACTED BY WATER*

Composition	Alfalfa 1	Alfalfa Brome	Alfalfa 2	Native Hay	Wheat Straw	Tame Hay
% Dry matter	28.4	26.0	23.6	20.4	3.9	25.2
% Ash	65.1	65.9	51.4	55.3	16.9	58.4
% Protein	37.8	41.9	38.3	29.2	45.5	39.8
% Calcium	55.2	63.1	36.0	54.0	23.5	48.6
% Phosphorus	2.5	5.4	2.7	3.3	10.0	5.8

* From Tables VII and VIII.

Small amounts of certain saccharides are also essential for optimum cellulolytic activity. The high water-soluble carbohydrate content of forage extracts should easily meet these requirements.

Summary:

1. Chemical analyses were conducted on 6 forages and their extracts.
2. The forage extracts were comprised essentially of carbohydrates, organic acids, protein (N X 6.25), and ash. Carbohydrate and organic acids accounted for 50% or more of the forage extract dry matter, with the remaining portion consisting mainly of protein and ash.
3. A considerable portion, 20% to 30%, of the dry matter content of the 5 hay samples was readily extracted by water. This extract contained over 50% of the ash, and approximately 30% and 40% of the protein content of the original forage. The amount of calcium extracted from the hays ranged from 36% to 63% while only 2.5% to 5.8% of total phosphorus was extracted.
4. Only 3.9% of the dry matter of wheat straw was extracted. Smaller fractions of the ash and calcium were extracted from wheat straw than from the 5 hays. However, larger portions

of the protein and phosphorus were extracted from wheat straw than from the 5 hays.

EXPERIMENT 3

It became evident, after a few fermentation runs, that replicate values were not reproducible when the media consisted of forage extracts. It was decided to study the source of this variation and to characterize *in vitro* fermentation as related to nutrient sufficiency and inoculum concentration.

Trial 1

Object: To determine the effect of varying concentrations of basal medium on *in vitro* cellulose digestion and to compare cellulose digestion values for the basal medium with those of other workers.

Experimental: The basal medium was used as described as well as at double and one-half this concentration.

The volume of final solution was 55 ml. except when the effect of dilution of the basal medium with 20 ml. of water was studied. The effect of adding 1 ml. of Na_2CO_3, instead of the customary 1.5 ml., to the basal medium was also determined.

A washed bacterial suspension was used. The digestion period was 24 hours.

Results and Discussion: The results for this trial are given in Table X.

In vitro cellulose digestion for the control medium compared favorably with the results reported by other workers using the same method. The 93.1% cellulose

TABLE X

EFFECT OF ALTERING NUTRIENT CONCENTRATION AND AMOUNT OF INOCULUM ON *IN VITRO* CELLULOSE DIGESTION

Treatment	Conc. of Basal Medium	Total Volume Solution	% Cellulose Digested	
			Mean	S.D.
Double basal medium	2	55	-3.9	4.2
Basal medium	1	55	93.1	0.6
Half basal medium	½	55	93.5	0.8
Basal medium with only 1 ml. of Na_2CO_3	1	55	93.0	1.7
Basal medium + 20 ml. additional water	1	75	92.1	0.4

digestion obtained in this trial is nearly the same as the 92% cellulose digestion reported by Donefer *et al*. (1960).

Doubling the concentration of the basal medium completely inhibited bacterial cellulolytic activity. The negative cellulose digestion values are probably attributable to the cellulose added in the bacterial suspension. These values were not determined for Trial 1 or 2 of this experiment, but were determined and used in the calculations for amounts of cellulose digested in subsequent experiments.

Halving the basal medium, the addition of only 1 ml. of Na_2CO_3 instead of 1.5 ml. to the basal medium or of 20 ml. of water to each fermentation tube did not

reduce in vitro cellulose digestion.

It appears that increasing the concentration of the basal medium beyond a certain level tends to inhibit in vitro cellulose digestion. The cause of this retardation was not established although high pH may have been responsible.

The concentration of the basal medium can be considerably reduced without reducing in vitro cellulose digestion.

Summary:

1. In vitro fermentation results are similar to those obtained by other workers.
2. Doubling the basal medium concentration completely inhibited in vitro cellulose digestion.
3. Halving the basal medium, reducing the amount of Na_2CO_3 from 1.5 ml. to 1 ml., and adding an additional 20 ml. of water to each fermentation tube did not alter in vitro cellulose digestion.

Trial 2

Object: To determine the effect of certain specific nutrients on in vitro cellulose digestion.

Experimental: Various nutrients were added either singly or in combination, at either two-thirds of basal medium levels or at basal medium levels. Rewashed bacterial suspension was used to minimize carry-over of soluble nutrients in the inoculum. A 44 hour fermentation period was used.

TABLE XI

IN VITRO CELLULOSE DIGESTION AS AFFECTED BY CERTAIN NUTRIENTS

Nutrients Added	% Cellulose Digested	
	Mean	S.D.
Basal medium	98.4	0.7
Na_2CO_3*	-3.5	0.3
$Na_2CO_3 + PO_4^{---} + SO_4^{--}$	-4.6	0.5
Na_2CO_3 + urea	-0.7	0.8
Na_2CO_3 + urea + $PO_4^{---} + SO_4^{--}$	-3.3	0.6
Na_2CO_3 + mineral mixture	40.5	0.6
Na_2CO_3 + mineral mixture + urea	87.9	2.6

* Na_2CO_3 maintained pH between 6.7 and 7.0 throughout the fermentation period.

S.D. Standard deviation.

Results and Discussion: The results of this trial are given in Table XI. The addition of Na_2CO_3; Na_2CO_3, PO_4^{---}, and SO_4^{--}; Na_2CO_3 and urea; or Na_2CO_3, urea, PO_4^{---}, and SO_4^{--} as the only nutrients to the artificial rumen did not promote *in vitro* cellulose digestion. The negative cellulose digestion values are probably attributable to cellulose added in the bacterial suspension.

When the nutrient medium consisted of Na_2CO_3 and mineral mixture, which contained Na^+, K^+, Mg^{++}, Cl^-, PO_4^{---}, and SO_4^{--}; or Na_2CO_3, mineral mixture, and urea, the

digestion of cellulose in vitro was 40.5% and 87.9% respectively.

It was concluded that a medium of Na_2CO_3 and mineral mixture is able to support measurable cellulose digestion. The supplementation of urea to the other nutrients supported even greater cellulose digestion.

Summary:

1. Cellulose digestion was not supported by Na_2CO_3, PO_4^{---}, SO_4^{--}, or urea, singly or in combination.
2. The amount of in vitro cellulose digestion supported by Na_2CO_3 and mineral mixture; or Na_2CO_3, mineral mixture, and urea, was 40.5% and 87.9%, respectively.

Trial 3

Object: To study the effect of washed inoculum concentration on in vitro cellulose digestion.

Experimental: Varying amounts of washed bacterial suspensions (2 to 30 ml.) were added to a constant nutrient medium and fermented for 24 hours. The two media used were water extracts of tame hay (20 ml. of forage water-soluble extract was used which was equivalent to the extract obtained from 1 gm. of forage) and of tame hay plus urea. The partial analyses of tame hay and tame hay extract are given in Tables VII and VIII, respectively. The media were buffered with Na_2CO_3. The total volume of liquid per tube was 55 ml. The amount of cellulose added with the

inoculum was determined.

Results and Discussion: The effect of varying levels of washed inoculum on *in vitro* cellulose digestion is illustrated in Figure 1. Because of a malfunctioning automatic pipette, duplicate *in vitro* cellulose digestion values were quite variable, nevertheless, a highly significant correlation was established. When the nutrient medium was inadequate, *in vitro* cellulose digestion was very highly correlated ($P < 0.01$) with concentration of washed bacterial suspension added. Coefficients of determination, r^2xy, between concentration of bacterial suspension added to either tame hay extract or tame hay extract plus urea and % cellulose digested were 0.990 and 0.941, respectively.

It appears that there is a carry-over of nutrients, when the bacterial suspension is washed only once, and that these nutrients supplement the basal medium, thereby increasing cellulose digestion. The improvement in cellulose digestion is related to concentration of bacterial suspension added.

Summary:

1. When the *in vitro* fermentation medium is deficient in certain essential components nearly all of the variation in cellulose digested is associated with the concentration of washed inoculum.

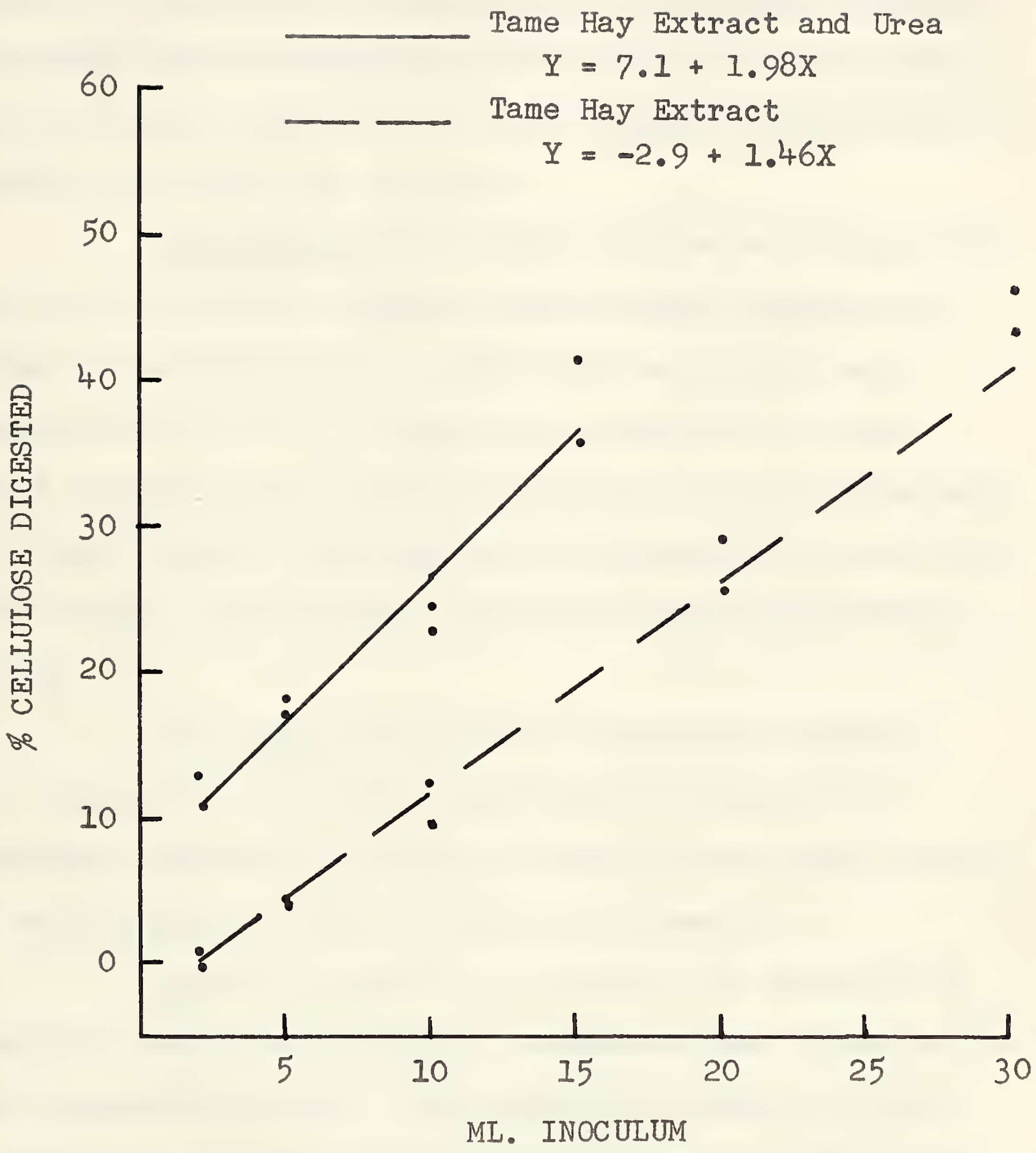

FIGURE 1. - Regression Curve for Ml. of Washed Inoculum and Cellulose Digested.

Trial 4

Object: To study the effect of rewashed bacterial suspension concentration on *in vitro* cellulose digestion and to determine if this effect is associated with bacterial population or with nutrient contaminants carried over with the bacteria.

Experimental: The basal medium was diluted to 1/6 and 1/4 of its original concentration, Na_2CO_3 was added to each dilution so that the final amount was equivalent to 1 ml. of Na_2CO_3 per fermentation tube. These diluted media, referred to as 1/6 basal medium and 1/4 basal medium, were used as the fermentation media for this trial. Final liquid volume per digestion tube was 55 ml.

The usual procedure for obtaining bacteria was duplicated to provide approximately double the bacterial population. The two samples were pooled, prior to centrifugation, and treated as one sample.

Varying amounts (2 to 30 ml.) of rewashed or rewashed autoclaved bacterial suspension were added to the two fermentation media. The centrifuge tubes, to which autoclaved rewashed bacterial suspension was added, were first inoculated with 5 ml. of live bacteria followed by the addition of autoclaved inoculum.

Results and Discussion: When an inadequate medium was used *in vitro* cellulose digestion was very

highly correlated ($P < 0.01$) with amount of rewashed bacterial suspension added to each fermentation tube. Coefficients of determination, r^2xy, between concentration of bacterial suspension added to either 1/4 basal medium or 1/6 basal medium and cellulose digested were 0.929 and 0.882, respectively.

The relationship between inoculum concentration and cellulose digestion, Figures 2 and 3, appeared to be curvilinear but since DF were small, regression curves were not determined.

The first 10 ml. increment of rewashed inoculum to the 1/6 and 1/4 basal medium increased cellulose digestion by 41.6% and 37.1%, respectively, while the second 10 ml. increment to these media supported an additional increase of only 11.5% and 6.2%, respectively.

The increase in cellulose digestion with the addition of 5, 10, or 15 ml. of either bacterial suspension to 1/4 basal medium, already inoculated with bacteria, was much greater in all cases with the live bacteria than with autoclaved inoculum. The addition of 20 ml. of autoclaved inoculum to the 1/4 basal medium increased *in vitro* cellulose digestion by 23.7% whereas the addition of 20 ml. of additional inoculum to the 1/4 basal medium increased *in vitro* cellulose digestion by 42.8%.

It appeared that when the fermentation medium

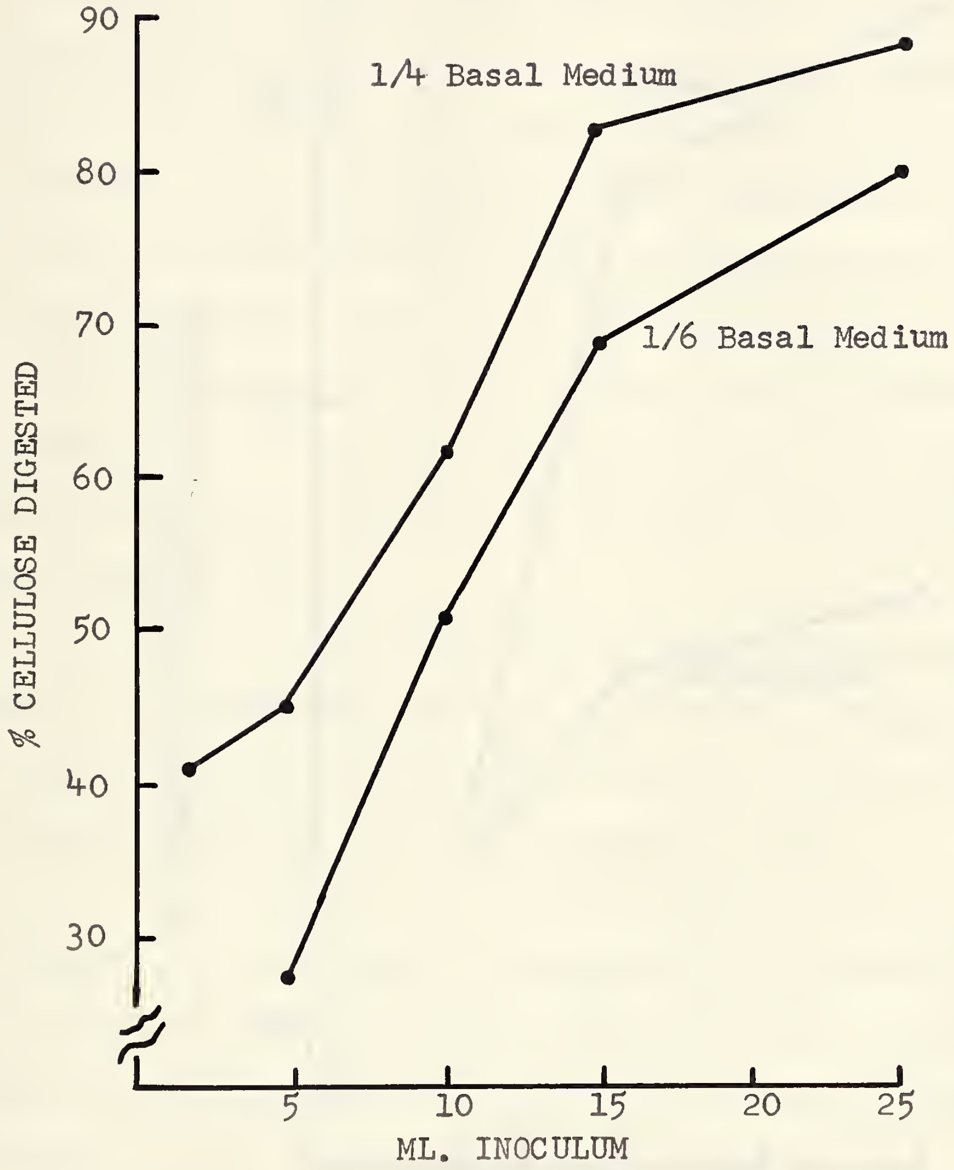

FIGURE 2. - Cellulose Digestion as Affected by Volume of Rewashed Inoculum

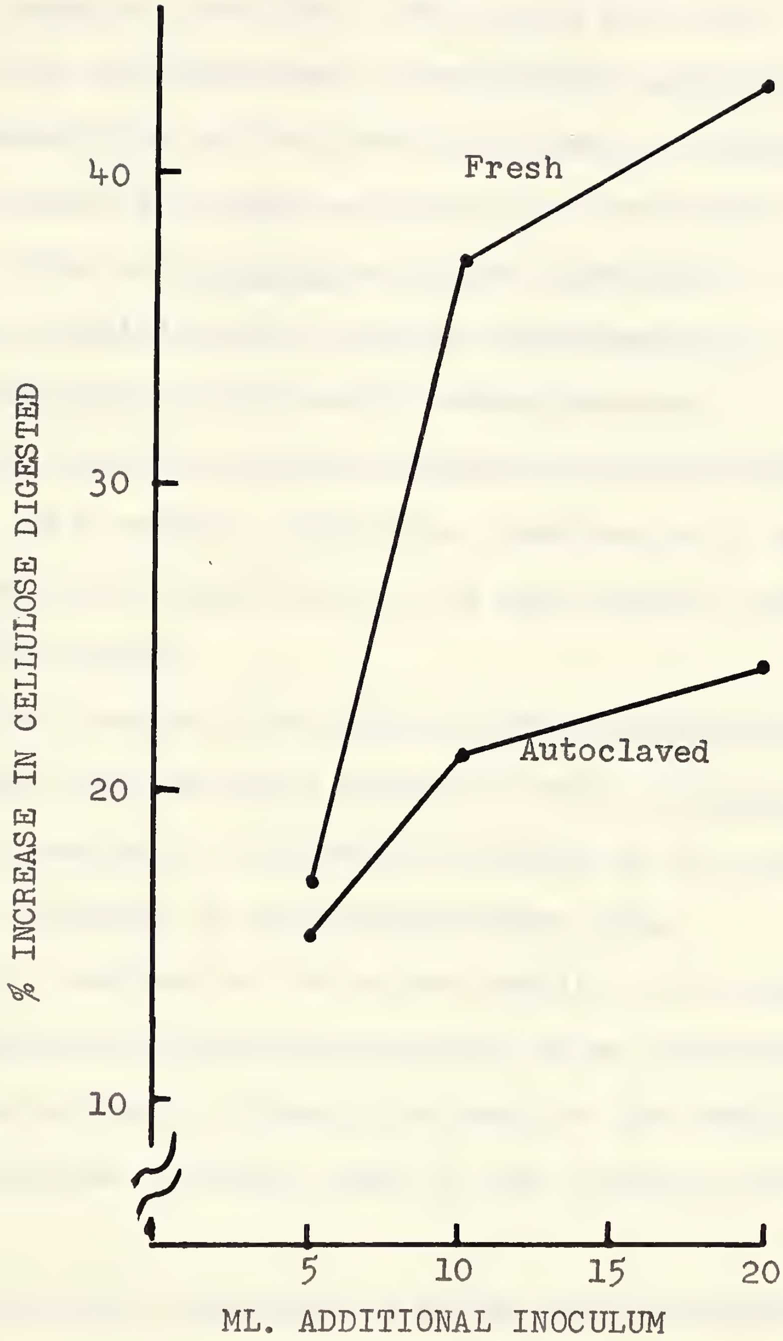

FIGURE 3. - Cellulose Digestion as Affected by Volume of Fresh or Autoclaved Rewashed Inoculum.

[illegible]

was inadequate, rewashed inoculum was about twice as effective in increasing cellulose digestion as was autoclaved rewashed inoculum. The reason for this difference was not determined. The greater cellulolytic bacterial population of the fresh inoculum, as compared to the autoclaved inoculum, may be one of the main factors influencing rate of *in vitro* cellulose digestion. Destruction, volatilization, and/or sequestering of essential nutrients, which would reduce nutrient availability, may have occurred during the autoclaving procedure. As a result, autoclaved inoculum would not stimulate cellulose digestion to the same extent that fresh inoculum would.

The results from this and other experiments indicate that when the basal medium is used, *in vitro* cellulose digestion is not greatly altered by the amount of inoculum included in each fermentation tube.

In contrast to the above results, the amount of *in vitro* cellulose digestion supported by an incomplete fermentation medium is directly related to the amount of washed or rewashed inoculum added to the digestion tube.

Summary:

1. When the fermentation media were inadequate, *in vitro* cellulose digestion was highly correlated ($P < 0.01$) with concentration of rewashed inoculum.
2. In vitro cellulose digestion was not greatly

affected by the concentration of inoculum when the basal medium was included in the fermentation tubes.

3. The cellulolytic activity of rewashed inoculum was about twice that of autoclaved rewashed inoculum. It was assumed that the lower cellulolysis in tubes containing rewashed autoclaved inoculum was associated with the smaller bacterial population and/or with a reduced availability of essential nutrients.

EXPERIMENT 4

Trial 1

Object: To determine the cellulolytic activity of aqueous extracts from forages of different quality.

Experimental: The fermentation medium consisted of 0.200 grams of forage extract dry matter and 1 ml. of Na_2CO_3 buffering agent. Volume per digestion tube was adjusted with distilled water to 50 ml., after which, 5 ml. of washed bacterial suspension was added. Twenty-four hour fermentation runs were replicated 3 times.

Results and Discussion: The results of 3 replicate fermentations to study the effects of aqueous extracts on cellulose digestion are given in Table XII. Analyses of variance and tests of significance for these data are presented in Tables XIII and XIV.

Differences between forages and replicates and the interaction between forage and replicates were highly significant ($P < 0.01$).

It was assumed that most of the significant differences ($P < 0.05$) between replicates was associated with variations in inoculum. The effect of inoculum concentration on *in vitro* cellulose digestion and possible causes of this effect are discussed in Experiment 3.

When compared on the basis of equal dry matter content, 4 out of 6 forage extracts supported essentially the same cellulose digestion. Per unit of dry matter extract, wheat straw extract was the most effective medium for the

TABLE XII

CELLULOLYTIC ACTIVITY OF FORAGE AQUEOUS EXTRACTS AND BASAL MEDIUM FOR 3 REPLICATES*

Replicate	Basal Medium	Alfalfa 1	Alfalfa Brome	Alfalfa 2	Native Hay	Wheat Straw	Tame Hay
1	93.4	59.4	59.4	53.3	66.2	68.2	33.2
2	94.1	32.9	32.9	39.7	28.6	55.0	12.3
3	94.7	25.4	27.1	21.5	20.1	37.8	3.4

* Each value is the average of duplicate determination

TABLE XIII

ANALYSIS OF VARIANCE OF THE DATA REPORTED IN TABLE XII

Source of Variation	DF	MS	F
Between replicates	2	3,066.6	22.8**
Between treatments	6	3,457.2	25.7**
Interaction	12	134.7	13.9**
Error	21	9.7	-

** Significant at $P < 0.01$

TABLE XIV

DUNCAN'S MULTIPLE - RANGE TEST OF SIGNIFICANCE*
Experiment 4 Trial 1

Mean cellulose digested - %

Medium

Tame Hay	Alfalfa 2	Native Hay	Alfalfa 1	Alfalfa Brome	Wheat Straw	Control
16.3	38.2	38.3	39.2	39.8	53.7	94.1

Replicate

1	2	3
32.9	42.2	61.9

* Means not underscored by the same line are significantly different from each other at the 5% level.

digestion of cellulose while tame hay extract was the poorest.

When the fermentation media consisted of forage extracts there was considerable variation between replicates in amount of cellulose digested; whereas when the basal medium was used there was only 1.3% difference between replicate means. This differential response between forage extract and the basal medium in replicate cellulose digestion is demonstrated by the highly significant interaction ($P < 0.05$).

Summary:

1. Forage aqueous extracts supported measurable cellulose digestion.
2. Wheat straw extracts and tame hay extracts respectively, provided the best and poorest media for the digestion of cellulose.
3. There was considerable variation between replicates in the extent of cellulose digestion supported by forage extracts. Replicate values for cellulolysis supported by the basal medium were relatively consistant. It was assumed that the variation between replicates when forage extracts were used was associated largely with variations in inoculum.

Trial 2

Object: To determine the cellulolytic activity of 6 forage extracts when supplemented with various nutrients.

Experimental: The fermentation medium consisted of the soluble nutrients from 1 gm. of forage to which Na_2CO_3 was added. The nutrients, used either singly or in combination to supplement the forage extracts, were Na_2CO_3, urea, PO_4^{---}, and SO_4^{--}.

Volume per digestion tube was adjusted to 50 ml. with distilled water, and 5 ml. of rewashed bacterial suspension served as inoculum.

Fermentation runs of 44 hour duration were replicated 4 times.

TABLE XV

EFFECT OF FORAGE EXTRACT AND SUPPLEMENT FOR 4 REPLICATES ON IN VITRO CELLULOSE DIGESTION

			Forage Extract					
Supplement	Replicate	Gm. TCA Protein*	Alfalfa 1	Alfalfa Brome	Alfalfa 2	Native Hay	Tame Hay	Wheat Straw
	1	0.0123	44.4	38.0	39.2	21.8	2.4	9.9
	2	0.0193	41.8	43.1	46.0	31.0	9.8	19.6
	3	0.0128	39.6	40.2	39.7	24.4	5.2	6.9
	4	0.0328	57.6	53.8	49.6	46.8	22.9	29.1
Urea	1	0.0122	40.2	40.8	41.9	64.0	32.5	19.1
	2	0.0193	56.0	50.3	55.7	68.6	37.2	20.4
	3	0.0128	35.8	39.1	33.7	45.4	20.8	7.3
	4	0.0328	58.7	55.5	50.5	79.8	46.2	35.2
Na_2HPO_4 $NaH_2PO_4H_2O$ Na_2SO_4	1	0.0123	81.5	59.4	61.0	20.2	8.1	14.0
	2	0.0193	73.8	70.6	88.1	27.4	13.2	21.9
	3	0.0128	76.5	65.0	80.0	28.0	12.3	12.5
	4	0.0328	84.7	86.1	95.4	61.2	27.3	45.2
Urea Na_2HPO_4 $NaH_2PO_4H_2O$ Na_2SO_4	1	0.0123	91.4	87.3	89.5	97.6	68.8	32.2
	2	0.0193	98.1	98.1	96.8	97.7	90.7	57.9
	3	0.0128	72.2	58.6	78.6	76.0	38.3	25.4
	4	0.0328	93.9	97.9	95,6	96.8	92.5	90.5

* Weight of bacterial protein added as inoculum to each fermentation tube.

TABLE XVI

ANALYSIS OF VARIANCE OF DATA FROM TABLE XV

Source of Variation	DF	M.S.	F
Between replicates	3	2,761.4	39.8**
Between forage extracts (F)	5	4,439.6	63.8**
1. Alfalfa vs. non-legumes	1	15,230.4	219.0**
2. Remainder	4	1,747.2	25.1**
Between minerals (M)	3	10,225.1	147.0**
Interaction F X M	15	623.0	9.0**
1. Alfalfa vs. non-legumes X mineral	3	2,339.0	33.6**
2. Remainder	12	193.5	2.8**
Error	69	69.5	

** Significant at $P < 0.01$

Results and Discussion: The amount of cellulose digested as affected by aqueous extract, mineral supplement, and replicate is given in Table XV. Analyses of variance and tests of significance of these data are presented in Tables XVI and XVII, respectively.

Differences between forage extracts, minerals, replicates, and interactions were highly significant ($P < 0.01$).

The replicate means were significantly different ($P < 0.05$). If the different replicate cellulose digestion

TABLE XVII

DUNCAN'S MULTIPLE - RANGE TEST OF SIGNIFICANCE*
Experiment 4 Trial 2

Mean cellulose digested - %

Forage Extracts

Wheat Straw	Tame Hay	Native Hay	Alfalfa Brome	Alfalfa 2	Alfalfa 1
28.0	33.0	55.4	61.5	65.1	65.4

Mineral Supplement

No Supplement	Urea	PO_4^{---} SO_4^{--}	Urea PO_4^{---} SO_4^{--}
31.8	43.1	50.6	80.1

Replicate

3	1	2	4
40.1	46.1	59.7	64.7

* Means not underscored by the same line are significantly different from each other at the 5% level.

values are associated with variations in bacterial population of the inoculum then a change of one factor should have resulted in a corresponding change in the other. Although the number of observations were few, the amount of bacterial

protein present in the inoculum tended to correspond somewhat with replicate cellulose digestion. Whether this was a cause and effect relationship it is not known, nevertheless it tends to confirm former observations that most of the variation between replicates was largely attributable to inoculum variation.

Significant differences ($P < 0.05$) between forage extracts are given in Table XVII.

The cellulolytic activity of forage extracts may be expressed in several ways. Obviously the validity of any comparison between forages depends on the method of expression used. This is illustrated by the calculations in Table XVIII.

The extract of wheat straw had the greatest cellulolytic activity on an equal extracted dry matter basis. However, on the basis of an equal amount of forage, wheat straw extract ranked 5th out of 6. Extracts of alfalfa 1 and 2, alfalfa brome, and native hay were no different on an equal extracted dry matter basis but the extracts of native hay had somewhat less cellulolytic activity per gram of forage because less dry matter was extracted from the material.

The extract of tame hay was very ineffective in supporting cellulose digestion and the more dry matter present in the extract, the lower the cellulolytic activity. This is suggestive of some imbalance of nutrients. Perhaps

TABLE XVIII

CALCULATED COMPARISON OF CELLULOLYTIC ACTIVITY OF FORAGE EXTRACTS *

		Alfalfa 1	Alfalfa Brome	Alfalfa 2	Native Hay	Wheat Straw	Tame Hay
1. D.M. Content of Extract	mg.	200	200	200	200	200	200
2. Cellulose Digested	mg.	78	80	76	77	107	33
3. Cellulose Digested per mg. D.M. (2/1)		0.39	0.40	0.38	0.38	0.54	0.16
4. D.M. Content of Extract from 1 gm. of Forage	mg.	260	240	220	180	40	230
5. Cellulose Digested per gm. of Forage	mg.	92	88	87	62	33	20
6. Cellulose Digested per mg. D.M. (5/4)		0.35	0.36	0.40	0.33	0.91	0.09

D.M. - Dry matter

* From Tables XII and XV

the relatively high soluble carbohydrate fraction of this extract may be involved.

On the basis of an empirical estimation of the expected relative animal response from these 6 forages, it would appear that, of the comparisons listed, those made on the basis of cellulose digested per unit of forage would be the most valid approximation of forage quality.

The addition of urea; PO_4^{---} and SO_4^{--}; or urea, PO_4^{---}, and SO_4^{--} to forage extracts, when compared to the unsupplemented forage extracts, significantly increased cellulose digestibility. This increase in cellulose digestibility would indicate that in some forage extracts the most limiting nutrients for cellulose utilization would be PO_4^{---}, SO_4^{--}, and urea; followed by PO_4^{---} and SO_4^{--}; and finally urea.

The cellulolytic activity of forage extracts, when supplemented with urea, PO_4^{---}, and SO_4^{--} was similar to the cellulolytic activity of mineral mixture and urea. Perhaps the level of nutrients was similar for the two media. If this is so it may be assumed that in addition to the nutrients reported in Table XIII some of the nutrients provided by the forage extract and required by rumen microflora for digestion of cellulose are K^+, Mg^{++}, and Cl^-.

The forage X mineral interaction was subdivided to include alfalfa vs. non-legume X mineral and remainder. The F value for the alfalfa vs. non-legume X mineral

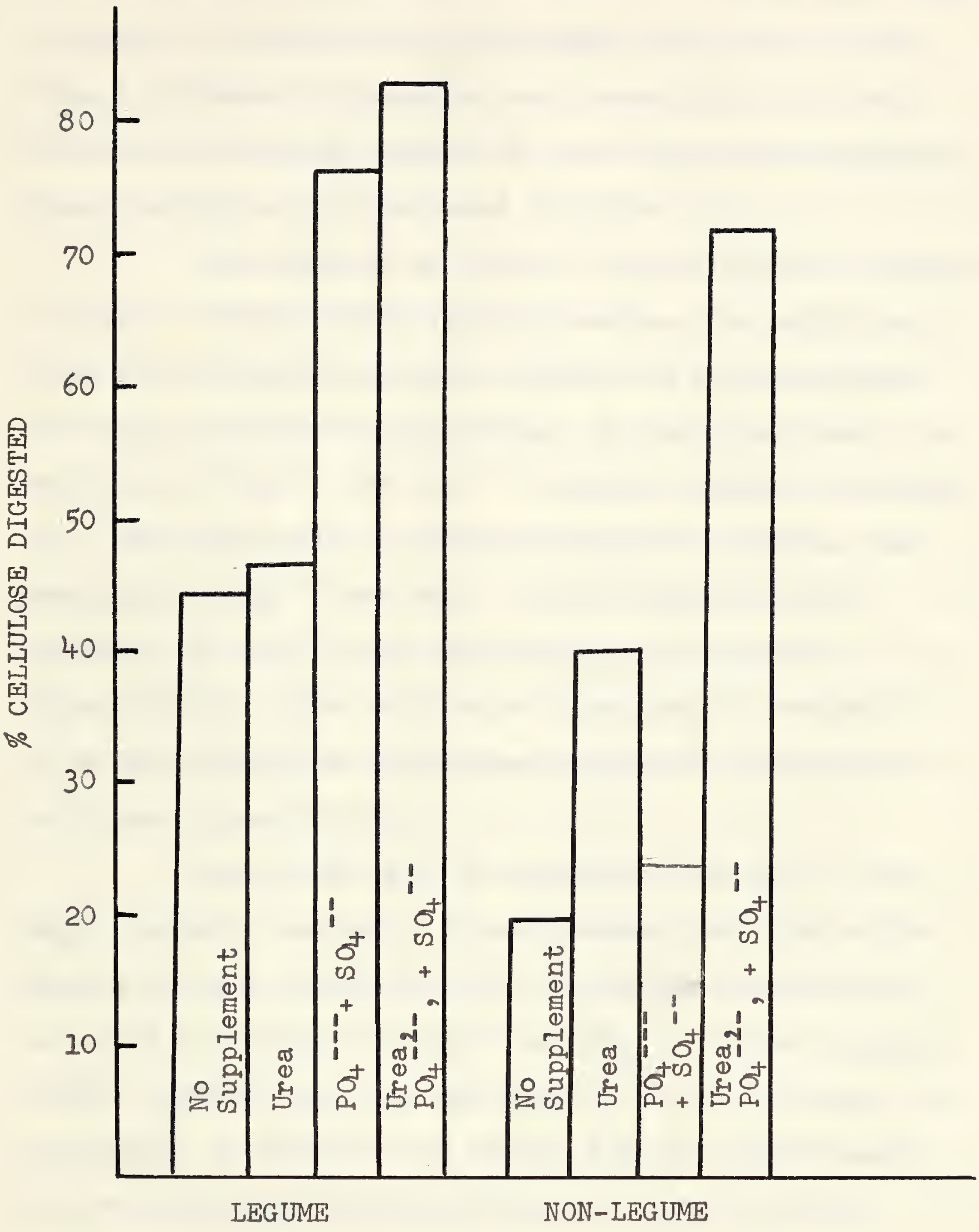

FIGURE 4. - Interaction Between Legumes vs. Non-Legumes and Mineral Supplement on in vitro Cellulose Digestion.

interaction is 12 times greater than the F value for the remainder. It was therefore assumed that most of the forage X mineral interaction was accounted for by the interaction between legumes vs. non-legumes and minerals. These results are illustrated in Figure 4.

The addition of urea to legume extracts improved cellulose digestibility slightly whereas the addition of urea to non-legume extracts resulted in a considerable increase in cellulose digestion. On the other hand, the addition of PO_4^{---} and SO_4^{--} to legume extracts resulted in a sharp increase in cellulose digestion whereas the addition of PO_4^{---} and SO_4^{--} to non-legume extracts resulted in only slight improvements in cellulose digestibility. The addition of urea, PO_4^{---} and SO_4^{--} to either legumes or non-legumes supported the highest cellulose digestibility.

The cation used to complement the PO_4^{---} and SO_4^{--} radicals was Na^+. It was assumed that the sodium cation was not responsible for any of the cellulolytic activity attributed to PO_4^{---} and SO_4^{--}. Hubbert _et al._ (1958) reported that the addition of 50 to 4,000 mcg. of sodium/ml. of fermentation medium did not significantly increase _in vitro_ cellulose digestion over a medium containing no sodium.

Summary:

1. The relative value of forage extracts in supporting

cellulose digestion varied greatly depending upon the method of expression of results. Cellulose digestion per unit of forage extracted seemed to be the most definitive method of those tried.

2. The supplementation of forage extracts with either urea; PO_4^{---} and SO_4^{--}; or PO_4^{---}, SO_4^{--} and urea significantly increased cellulose digestion.
3. The addition of urea to legume extracts improved cellulose digestibility slightly, whereas the addition of urea to non-legume extracts resulted in a considerable increase in cellulose digestion. On the other hand, the addition of PO_4^{---} and SO_4^{--} to legume extracts resulted in a sharp increase in cellulose digestion, whereas the addition of PO_4^{---} and SO_4^{--} to non-legume extracts resulted in only slight improvements in cellulose digestibility. The addition of urea, PO_4^{---}, and SO_4^{--} to either legume extracts or non-legume extracts produced the greatest improvement in cellulose digestion.

EXPERIMENT 5

Object: To repeat the procedure of Experiment 4 using another set of forages.

Description of Forages: Eight forages supplied by the Agricultural Soils and Feed Testing Laboratory, University of Alberta, were used. The chemical composition of these forages is given in Table XIX.

Experimental: The experimental procedure was identical to that reported in Experiment 4, Trial 1, except that the extract from a smaller amount of forage was used, digestion period was 24 hours, and a washed bacterial suspension used. The water-soluble nutrients from 0.700 grams of forage, instead of from 1.000 grams of forage, was used for the fermentation medium. This modification was made because of the observation that the amount of cellulose in the 0.700 grams of forage is approximately equal to the amount of cellulose included in each fermentation tube. Therefore, presumably, the extent of in vitro cellulose digestion in the presence of a certain forage extract might be an indication of that forages' ability to supply nutrients for digestion of its own cellulose.

Results and Discussion: The amount of cellulose digested as affected by aqueous extract and mineral supplement for 2 replicates is given in Table XX. Analyses of variance and tests of significance of these data are presented in Tables XXI and XXII.

TABLE XIX

CHEMICAL COMPOSITION OF 8 FORAGES

Number, Name & Description	% Moisture after Drying	% Moisture before Drying	Protein %		% Crude Fiber	% Calcium	% Phosphorus
			Air Dry Basis	Moist Free Basis			
1. Alfalfa-oats silage, Odor fair, color good	9.2	76.6	2.8	12.1	7.6	0.43	0.06
2. Mixed grass legume	9.5	9.5	12.6	13.9	27.1	1.43	0.23
3. Sweet clover, hay mature, color fair, odor good	7.1	7.1	13.4	14.4	35.6	0.92	0.27
4. Alsike clover, musty & moldy, odor poor	14.3	14.3	12.2	14.2	27.9	1.27	0.14
5. Oat silage, color good, odor fair	5.0	66.7	3.8	11.4	9.7	0.02	0.07
6. Grass hay, coarse, color fair	7.4	7.4	7.8	8.4	30.4	0.28	0.10
7. Oat green feed, mature, color fair	9.4	9.4	10.1	11.1	29.9	0.16	0.22
8. Spring growth Russian Wild Rye	7.5	7.5	27.1	29.2	19.5	0.35	0.35

TABLE XX

PER CENT CELLULOSE DIGESTED AS AFFECTED BY FORAGE EXTRACT, SUPPLEMENT, AND REPLICATE

		Forage Extract*							
Supplement	Replicate	Alfalfa	Mixed Grass Legume	Sweet Clover Hay	Alsike Clover	Oat Silage	Grass Hay Coarse	Oat Green Feed	Russian Wild Rye
	1	33.9	13.2	28.2	27.9	42.6	13.7	29.4	37.6
	1	32.6	15.0	24.0	23.4	40.8	15.3	23.2	39.5
	2	48.7	35.5	39.0	29.2	40.8	19.5	27.9	43.4
	2	41.0	30.5	31.8	26.6	46.3	18.2	25.5	45.5
Urea	1	33.2	19.5	34.7	25.3	48.7	21.6	25.8	46.0
	1	32.1	16.6	26.6	22.1	46.8	21.3	30.8	40.8
	2	47.1	30.8	31.8	27.4	50.5	25.8	35.3	46.3
	2	43.9	32.1	36.8	31.0	54.7	29.7	34.2	46.8
Na_2HPO_4 $NaH_2PO_4.H_2O$ & Na_2SO_4	1	62.6	34.8	46.6	35.0	66.0	18.8	42.1	71.3
	1	56.6	35.3	39.2	31.3	68.4	23.4	47.6	73.7
	2	70.3	50.0	57.1	37.4	70.5	29.5	52.1	80.5
	2	73.4	47.6	60.3	33.7	76.8	32.6	55.3	75.0
Na_2HPO_4 $NaHPO_4.H_2O$ Na_2SO_4 & Urea	1	83.7	67.6	69.7	46.9	68.9	39.7	68.4	73.9
	1	82.9	63.7	71.8	55.8	73.7	44.7	66.6	76.6
	2	91.2	91.8	87.1	85.5	90.3	75.8	91.8	92.9
	2	92.6	87.0	94.6	80.8	92.6	85.3	91.3	87.6

* Oven-dried alfalfa silage and oven-dried oat silage were used for preparation of forage extracts.

TABLE XXI

ANALYSIS OF VARIANCE OF DATA FROM TABLE XX

Source of Variation	D.F.	M.S.	F
Between replicate (R)	1	42,136.2	4,580.0**
Between forage (F)	7	1,862.1	202.0**
Between mineral (M)	3	14,400.6	1,570.0**
Interaction (R X F)	7	44.2	4.8**
Interaction (F X M)	21	129.0	14.0**
Interaction (M X R)	3	127.1	13.8**
Interaction (M X R X F)	21	86.4	9.4**
Error	64	9.2	

** Significant at $P < 0.01$.

Differences between replicates, forage extracts, and minerals and all interactions were highly significant ($P < 0.01$).

It was assumed that the highly significant ($P < 0.01$) replicate X forage and replicate X mineral interaction was mainly caused by the variable nature of replicate inoculum. The 2 replicate means were highly significantly different ($P < 0.01$).

The legumes in this experiment, in most cases, were inferior in apparent quality to the legumes used in the former experiment, while the apparent quality of most

TABLE XXII

DUNCAN'S MULTIPLE - RANGE TEST OF SIGNIFICANCE*
Experiment 5

Mean cellulose digested - %

Forage Extracts

Grass Hay Coarse	Alsike Clover	Mixed Grass Legume	Oat Green Feed	Sweet Clover Hay	Alfalfa Silage	Russian Wild Rye	Oat Silage
32.2	38.7	41.9	46.7	48.7	57.9	61.1	61.2

Mineral Supplement

No Supplement	Urea	PO_4^{---} SO_4^{--}	Urea PO_4^{---} SO_4^{--}
30.9	34.2	51.7	77.3

Replicate

1	2
42.3	54.3

* Means not underscored by the same line are significantly different from each other at the 5% level.

of the non-legumes was much superior to those used in the other experiments. This could partially account for the failure to demonstrate the significant interaction between legumes vs. non-legumes X mineral that was observed in Experiment 4.

The interaction between forage extract and

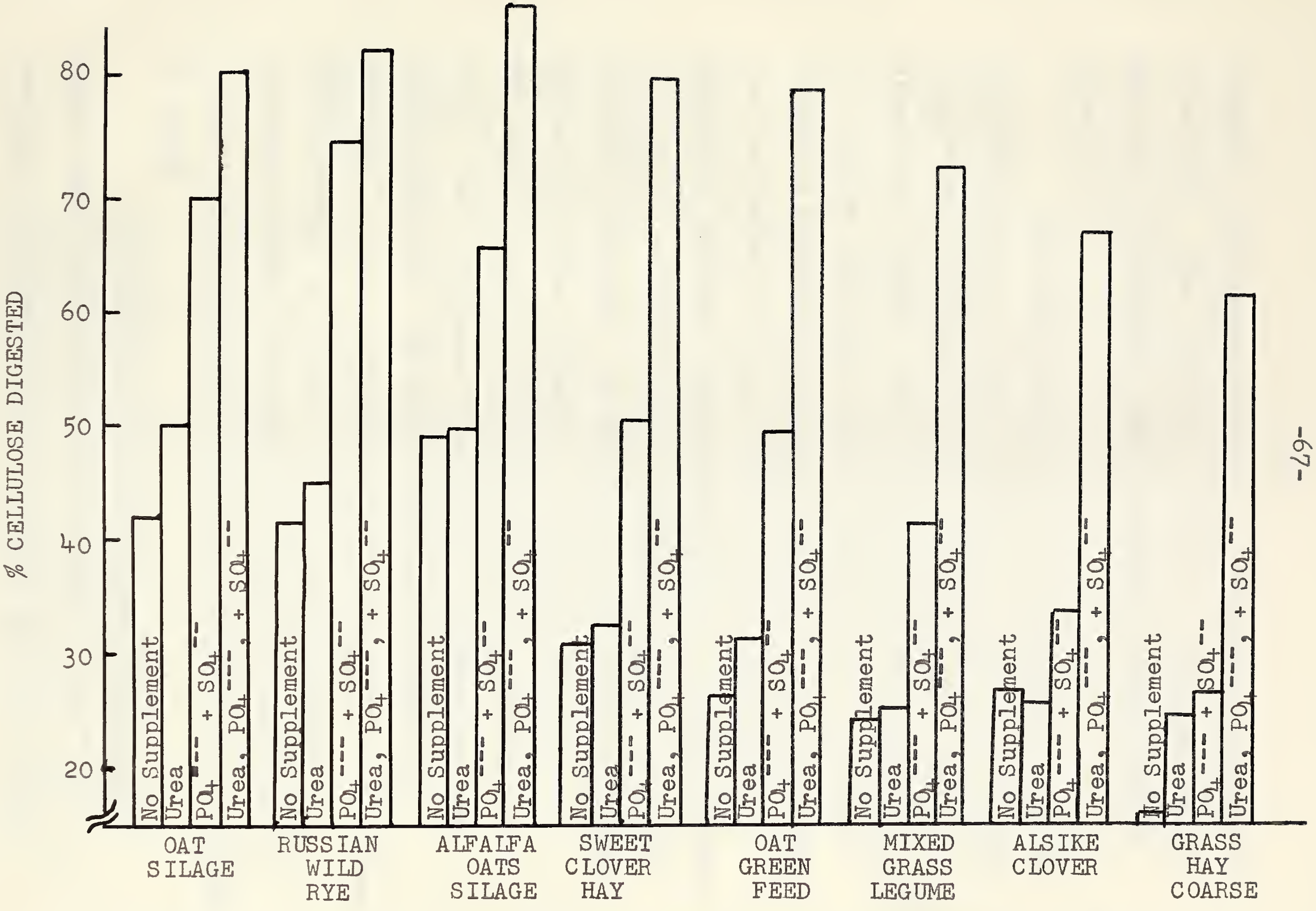

FIGURE 5. - Interaction Between Forage Extracts and Supplement on *in vitro* Cellulose Digestion.

mineral, illustrated in Figure 5, was highly significant ($P < 0.01$). The interaction observed in this experiment was not as pronounced as that observed in Experiment 4, Trial 2.

The data from Table XXII show that urea, as the only supplement to forage extracts, was quite ineffective, supporting an average of only 3.3% increase in cellulose digestion. However, the supplementation of this nutrient to a medium containing forage extract, PO_4^{---}, and SO_4^{--} caused an increase in cellulose digestion of 25.6% above that supported by forage extract, PO_4^{---}, and SO_4^{--}. Similarly, although the supplementation of forage extracts with PO_4^{---} and SO_4^{--} gave an average increase of 20.8% cellulose digested, the addition of these ions to forage extract plus urea supported an increase of 43.1% over the cellulose digestion supported by forage extract plus urea. These increases in cellulose digestion are not merely additive. The single addition of either PO_4^{---} and SO_4^{--} or urea to forage extracts resulted in an average total increase of (20.8% + 3.3%) 24.1% in cellulose digestion whereas the addition of all three nutrients to forage extracts increased cellulose digestion by an average of 46.4%.

It would seem that this apparent synergistic effect developed because levels of PO_4^{---}, SO_4^{--} and available nitrogen of forage extracts were only barely

marginal for cellulolytic activity. The inclusion of any one nutrient would increase cellulose digestion only to the point where some other nutrient became limiting. Thus supplementation of forage extracts with either PO_4^{---} and SO_4^{--} or urea increased cellulose digestion somewhat but the increase in cellulose digestion observed when all three nutrients were included in the medium was much greater than the sum of the increase when they were added singly.

On the basis of these results it is assumed that the water-soluble constituents of forages are able to support measurable cellulose digestion and that these constituents, in most cases, when supplemented with urea, PO_4^{---}, and SO_4^{--} provide an almost complete medium for the digestion of forage cellulose. The actual extent of cellulose digestion of a given forage would, however, depend on various characteristics of the forage itself such as the degree of lignification.

Summary:

1. The extracts of the 8 forages described possessed measurable cellulolytic activity. Supplementation with PO_4^{---} and SO_4^{--} or urea gave some cellulolytic response whereas supplementation with all these factors supported cellulose digestion almost equal to that supported by the basal medium.

2. The differential responses with the extracts of legumes vs. non-legumes to supplementation which was observed in Experiment 4 was not as consistent in this trial, but similar trends were observed.

CONCLUSIONS

It was observed that, even though standard conditions were followed in the preparation of washed and rewashed suspension of rumen microorganisms, variable *in vitro* results were obtained between replicates if the levels of nutrients in the fermentation medium were insufficient for rapid cellulose digestion. In studies of the adequacy of a particular medium for *in vitro* cellulose digestion, this variation due to inocula should be controlled by biological or statistical techniques.

It was demonstrated that forage aqueous extracts are able to support measurable cellulose digestion. Supplementation with PO_4^{---}, SO_4^{--}, and urea supported cellulose digestion almost equal to that supported by the basal medium. Further studies of aqueous extracts involving the order in which nutrients become limiting for optimum cellulose digestion may provide information concerning the mechanisms of cellulose digestion as well as about the forage itself. Also, research into nutrient imbalances could be conducted using aqueous extracts of forages as the medium.

There were considerable variations between forage aqueous extracts and probably forages themselves in cellulolytic activity. Therefore, when forages are evaluated *in vitro*, some consideration should be given to the ability of forages to promote their own digestion. Such studies would also require an assessment of the

contribution of saliva, nutrient residues in the rumen, and fermentation by-products.

It is also suggested that the artificial rumen technique can be used as a screening technique to determine the sufficiency of supplementary nutrients for maximum forage utilization and the expected *in vivo* response when they are added to the forage.

Further studies of the aqueous extracts of forage should yield valuable information concerning the nature and mechanism of the fermentation process in the rumen, and should be one additional means whereby forages may be characterized as to their ultimate value to the ruminant animal.

BIBLIOGRAPHY

Anderson, R., E. Cheng, and W. Burroughs. 1956. A laboratory technique for measuring phosphorus availability of feed supplements fed to ruminants. J. Animal Sci. 15:489.

Annison, E.F., 1954. Some observations on the volatile fatty acids in the sheep's rumen. Biochem. J. 57:400.

Annison, E.F., and D. Lewis. 1959. Metabolism in the rumen. John Wiley and Sons Inc. New York.

Arias, C., W. Burroughs, P. Gerlough, and R.M. Bethke. 1951. The influence of different amounts and sources of energy upon in vitro urea utilization by rumen microorganisms. J. Animal Sci. 10:683.

Asplund, J.M., R.T. Berg, L.W. McElroy, and W.J. Pigden. 1958. Dry matter loss and volatile fatty acid production in the artificial rumen as indices of forage quality. Canadian J. Animal Sci. 38:171.

Association of Official Agricultural Chemists. 1955. "Official Methods of Analysis of the Association of Official Agricultural Chemists." 8th Ed. Washington, D.C.

Barnett, A.J.G., and R.L. Reid. 1957. Studies on the production of volatile fatty acids from grass by rumen liquor in the artificial rumen. 1. The volatile acid production from fresh grass. J. Agr. Sci. 49:315.

Belasco, I.J., 1954. Comparison of urea and protein meals as nitrogen sources for rumen microorganisms: The production of volatile fatty acids. J. Animal Science. 13:748.

Belasco, I.J., 1956. The role of carbohydrate in urea utilization cellulose digestion and fatty acid formation. J. Animal Sci. 15:496.

Bentley, O.G., R.R. Johnson, S. Vanecho, and C.H. Hunt. 1954. Studies in factors needed by rumen microorganisms for cellulose digestion in vitro. J. Animal Sci. 13:581.

Bentley, O.G., R.R. Johnson, T.V. Hershberger, J.H. Cline, and A.L. Moxon. 1955. Cellulolytic-factor activity of certain short-chain fatty acids for rumen microorganisms in vitro. J. Nutrition. 57:389.

Bryant, M.P. and L.A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36:205.

Bryant, M.P., and R.N. Doetsch. 1955. Factors necessary for the growth of Bacteroides succinogenes in the volatile acid fraction of rumen fluid. J. Dairy Sci. 38:340.

Bryant, M.P. 1959. Observations on the nutrition of Bacteroides succinogenes. A ruminal cellulolytic bacterium. J. Dairy Sci. 42:1831.

Burroughs, W., N.A. Frank, P. Gerlough, and R.M. Bethke. 1950. Preliminary observations upon factors influencing cellulose digestion by rumen microorganisms. J. Nutr. 40:9.

Burroughs, W., A. Latona, P. De Paul, P. Gerlough, and R.M. Bethke, 1951. Mineral influences upon urea utilization and cellulose digestion by rumen microorganisms using the artificial rumen technique. J. Animal Sci. 10:693.

Cason, J.L., R.D. Mochrie, and W.E. Thomas, 1955. Self containing cellulose digesters of common ruminant feeds. J. Animal Sci. 14:1236 (Abstr.).

Cheng, E.W., G. Hall, and W. Burroughs. 1955. A method for the study of cellulose digestion by washed suspensions of rumen microorganisms. J. Dairy Sci. 38:1255.

Cline, H.J., T.V. Hershberger, and O.G. Bentley. 1958. Utilization and/or synthesis of valeric acid during the digestion of glucose, starch, and cellulose by rumen microorganisms in vitro. J. Animal Sci. 17:284.

Crampton, E.W. And L.A. Maynard. 1938. The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutr. 15:383.

Davy, L.A., G.C. Cheeseman, and C.A.E. Briggs. 1960. Evaluation of an improved artificial rumen designed for continuous control during prolonged operation. J. Agric. Sci. 55:2.

Dehority, B.A., K. El-Shazly, and R.R. Johnson. 1960. Studies with cellulolytic fraction of rumen bacteria obtained by differential centrifugation. J. Animal Sci. 19:1098.

Deriaz, R.E. 1961. Routine analysis of carbohydrates and lignin in herbage. J. Sci. Food Agric. 12:152.

Donefer, E., E.W. Crampton, and L.E. Lloyd. 1960. Prediction of the nutritive value index of a forage from in vitro rumen fermentation data. J. Animal Sci. 19:545.

Duncan, D.B. 1955. Multiple range and multiple F tests. Biometrics. 11:1.

Elsden, R.S. 1946. The application of silica gel partition chromatogram to the estimation of volatile fatty acids. Biochem. J. 40:252.

Gall, L.S., S.E. Smith, D.E. Becher, C.N. Stark, and J.K. Loosli, 1949. Rumen bacteria in cobalt deficient sheep. Science 109:468.

Gray, F.V., A.F. Pilgrim, and R.A. Weller. 1951. Fermentation in the rumen of the sheep. 1. The production of volatile fatty acids and methane during the fermentation of wheaten hay and lucerne hay _in vitro_ by microorganisms from the rumen. J. Exp. Biol. 28:74.

Hash, J.H. and K.W. Kling. 1954. Demonstration of an oligosaccharide intermediate in the enzymatic hydrolysis of cellulose. Science 120:1033.

Hastings, E.G. 1944. The significance of the bacteria and protozoa of the rumen of the bovine. Bacteriol Revs. 8:235.

Hershberger, T.V., O.G. Bentley, J.H. Cline, and W.G. Tyznik. 1956. Formation of short-chain fatty acids from cellulose, starch, and metabolic intermediates by ovine and bovine rumen microorganisms. J. Agr. Food Chem. 4:952.

Hirst, E.L., D.J. MacKenzie, and C.B. Wylam. 1959. Analytical studies on the carbohydrates of grasses and clovers. lX. -- Changes in carbohydrate composition during the growth of lucerne. J. Sci. Food Agric. 10:19.

Howard, B.H. 1955. Hydrolysis of the soluble pentosans of wheat flour and _Rhodymenia palmata_ by ruminal microorganisms. Biochem. J. 60:1.

Hubbert, F. Jr., E. Cheng, and W. Burroughs. 1958. Mineral requirements of rumen microorganisms for cellulose digestion _in vitro_. J. Animal Sci. 17:559.

Hubbert, F. Jr., E. Cheng, and W. Burroughs. 1958b. The influence of potassium, sodium, rubidium, lithium, and cesium on _in vitro_ cellulose digestion by rumen microorganisms with observations upon sodium and potassium influences in lamb fattening rations. J. Animal Sci. 17:577.

Huhtanen, C.N., R.K. Saunders, and L.S. Gall. 1954. Fiber digestion using the miniature artificial rumen. J. Dairy Sci. 37:328.

Jackson, M.L., 1958. Soil Chemical Analysis. Prentice Hall Inc. Englewood Cliffs, N.J.

Johnson, R.R., B.A. Dehority, and O.G. Bentley. 1958. Studies on the in vitro rumen procedure. 1. Improved inoculum preparation and the effects of volatile fatty acids on cellulose digestion. J. Animal Sci. 17:841.

Kamstra, L.D., A.L. Moxon, and O.G. Bentley. 1958. The effect of stage of maturity and lignification on the digestion of cellulose in forage plants by rumen microorganisms in vitro. J. Animal Sci. 17:199.

Kitts, W.D., and L.A. Underhofler. 1954. Hydrolytic products of cellulose and cellulolytic enzymes. J. Agr. Food Chem. 2:639.

Kleiber, M., H.H. Cole, and S.W. Mead, 1943. Bloat in cattle and composition of rumen gases. J. Dairy Sci. 26:929.

Kon, S.K., and J.W.G. Porter, 1947. The synthesis of vitamins in relation to requirements. Nutr. Abstr. Rev. 17:31.

Louw, J.G., H.H. Williams, and L.A. Maynard. 1949. A new method for the study in vitro of rumen digestion. Science 110:478.

McDonald, W.I. 1952. The role of ammonia in ruminal digestion of protein. Biochem. J. 51:86.

Marston, H.R. 1948. The fermentation of cellulose in vitro by organisms from the rumen of sheep. Biochem. J. 42:564.

Mills, R.C., A.N. Booth, G. Bohsted, and E.B. Hart. 1942. The utilization of urea by ruminants as influenced by the presence of starch in the ration. J. Dairy Sci. 25:925.

Morris, D.L. 1948. Quantitative determination of carbohydrate with Dreywood's anthrone reagent. Science 107:255.

Neish, A.D. 1952. Analytical Methods for Bacterial Fermentations. National Research Council (Canada) Report No. 46-8-3 (Second Revision).

Pearson, R.M., and J.A.B. Smith. 1943. The utilization of urea in the bovine rumen. III. Synthesis and breakdown of protein in rumen ingesta. Biochem. J. 37:153.

Quicke, G.V., O.G. Bentley, H.W. Scott, and A.L. Moxon, 1959. Cellulose digestion in vitro as a measure of digestibility of forage cellulose in ruminants. J. Animal Sci. 16:502.

Ruf, E.W., W.H. Hale, and W. Burroughs, 1953. Observations upon an unidentified factor in feedstuffs stimulatory to cellulose digestion in the rumen and improved liveweight gains in lambs. J. Animal Sci. 12:731.

Shaw, J.C. 1959. Nutritional physiology of the rumen: A new approach to rumen nutrition. Feedstuffs 31 (37):18.

El-Shazly, K., B.A. Dehority, and R.R. Johnson. 1960. A comparison of the all-glass, semi-permeable membrane, and continuous flow types of apparatus for in vitro rumen fermentations. J. Dairy Sci. 43:1455.

Snedecor, G.W. 1956. Statistical Methods Applied to Experiments in Agriculture and Biology. (5th Ed.) The Iowa State College Press, Ames, Iowa.

Stewart, W.E., D.G. Stewart, and L.H. Schultz. 1958. Rates of volatile fatty acid production in the bovine rumen. J. Animal Sci. 17:723.

Swift, R.W., R.L. Cowan, G.P. Barren, K.H. Maddy, and E.C. Grese. 1951. The effect of alfalfa ash upon roughage digestion in the sheep. J. Animal Sci. 10:434.

Thomas, G.J. 1960. Metabolism of the soluble carbohydrates of grasses in the rumen of sheep. J. Agric. Sci. 54:360.

Wasserman, R.H., C.W. Duncan, E.S. Churchill, and C.F. Huffman. 1952. The effect of antibiotics on in vitro cellulose digestion by rumen microorganisms. J. Dairy Sci. 35:571.

Waite, R. 1957. The water-soluble carbohydrates of grasses. III. First and second year growth. J. Sci. Food Agric. 8:422.

Waite, R. and A.R.N. Gorrod. 1959a. The structural carbohydrates of grasses. J. Sci. Food Agric. 10:308.

Waite, R. and A.R.N. Gorrod. 1959b. The comprehensive analysis of grasses. J. Sci. Food Agric. 10:317.

Wright, D.E. 1959. Pectic enzymes in rumen protozoa. Arch. Biochem. Biophys. 86:251.

B29794